Respiratory burst activity of intestinal macrophages in normal and inflammatory bowel disease

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SUMMARY  Macrophages isolated from normal mucosa (greater than 5 cm from tumour) and inflamed mucosa (from patients with inflammatory bowel disease) of colon and ileum were studied for their ability to undergo a respiratory burst as assessed by reduction of nitroblue tetrazolium to formazan. Using phorbol myristate acetate (PMA) and opsonised zymosan as triggers, only a minority (median: 8% for zymosan and 9% for PMA) of macrophages isolated from normal colonic mucosa demonstrated release of oxygen radicals. In contrast, a significantly greater (median: 17% for zymosan and 45% for PMA) proportion of macrophages isolated from inflamed colonic mucosa were able to undergo respiratory burst. Studies with normal and inflamed ileum showed similar results. Stimulation of macrophages isolated from normal colon with interferon-γ produced only a small increase in the proportion of cells showing release of oxygen radicals. We conclude that the respiratory burst capacity of majority of macrophages isolated from normal colon and ileum is downregulated and a greater proportion of macrophages isolated from inflamed colon and ileum are able to undergo a respiratory burst.

An activated macrophage is capable of mediating antimicrobial and cytotoxic effects. The activation process is accompanied by a number of physiological and biochemical changes. These include enhanced secretion of neutral proteases, acid hydrolases, enhanced expression of HLA class II molecules, down regulation of receptors for mannose terminated glycoconjugates and an enhanced or upregulated respiratory burst. During a respiratory burst, oxygen is taken up by the cell and enzymatically converted by membrane associated NADPH oxidase to superoxide anion (O₂⁻), hydrogen peroxide (H₂O₂) and hydroxyl radical (OH·). A respiratory burst can be triggered by phagocytosis of particles such as zymosan or by soluble surface active agents such as phorbol myristate acetate (PMA). Some of the reactive metabolites of oxygen (RMO) can be released outside the cell where damage to surrounding cells can occur.

Ulcerative colitis and Crohn’s disease are chronic inflammatory disorders of unknown aetiology, and macrophages are increased in number in the mucosa of both being especially prominent in granulomata of Crohn’s disease. There is increased monocyte turnover and activation in active disease and these cells are likely to be the main source of the intestinal macrophages. Currently, there is very little information on the function of macrophages in normal and inflamed intestine.

In this study, macrophages isolated from histologically normal colon or ileum and from the colon and ileum of patients with active inflammatory bowel disease (IBD) were studied for their ability to undergo a respiratory burst. Opsonised zymosan, phorbol myristate acetate (PMA) and N-formylmethionyl-leucyl-phenylalanine (FMLP) were used as triggers. Respiratory burst activity was assessed by reduction of nitroblue tetrazolium to formazan. In some studies, the effect of interferon-gamma (IFN-γ) and lipopolysaccharide (LPS) on respiratory burst capacity of isolated macrophages was studied.

Methods

STUDY POPULATION
Patients (10 men, 14 women, age 23–83 years)
undergoing colonic resection for carcinoma (16), familial polyposis (two), idiopathic constipation (one) or right hemicolectomy for caecal carcinoma (five) were studied.

Thirty two patients (18 men, age 14–70 years) with inflammatory bowel disease involving the colon and 12 patients (seven men, age 16–71 years) with ileal Crohn’s disease were studied. All patients undergoing intestinal resection for inflammatory bowel disease had active disease. All except two (with colonic Crohn’s disease) were receiving intravenous hydrocortisone.

**CELL ISOLATION**

Normal mucosa from patients without inflammatory bowel disease was obtained at least 5 cm from tumour and was macroscopically as well as histologically normal. Inflamed, and in some cases non or minimally inflamed mucosa was studied from ulcerative colitis or Crohn’s disease colon or ileum. Mononuclear cells were isolated by a modification of the technique described by Bull and Bookman.²²

The mucosa was transported in cold (4°C) RPMI 1640 (Gibco) and extensively washed with Hanks’ Balanced Salt Solution without calcium and magnesium (CMF HBSS; Flow laboratories). 5–10 mm strips of mucosa were dissected and incubated with 1 mmol dithiothreitol (Sigma) for 20 min at room temperature (with shaking every five minutes). Epithelial cells were removed by shaking the mucosa with 5 mM EDTA (BDH Chemicals, Poole, England) at 37°C followed by washing with CMF-HBSS. This was repeated twice and after the last EDTA step, washing was performed with Hanks Balanced Salt Solution with calcium and magnesium (HBSS; Flow laboratories). The mucosa was cut into 2 mm pieces and digested with collagenase (from Clostridium histolyticum; Boehringer Mannheim) at a concentration of 100 mg/100 ml culture medium (10% fetal calf serum in RPMI 1640; Gibco) with shaking for three hours at 37°C. After digestion, the cells were filtered through a 100 μ nylon mesh and extensively washed with HBSS before centrifugation on Ficoll-Paque (Pharmacia) to obtain mononuclear cells. After further washing, the mononuclear cells were resuspended in culture medium. To determine the proportion of macrophages present, cyt centrifuge preparations of the mononuclear cells were made and stained with macrophage specific monoclonal antibody Y1/82A using the peroxidase technique.²³

The respiratory burst activity was assayed within two hours of isolation (except when the mononuclear cells were stimulated with IFN-γ and LPS). All the media contained 100 U/ml penicillin, 100 μg/ml streptomycin and 5 μg/ml gentamycin.

**TRIGGERS**

Phorbol 12-myristate 13-acetate (PMA; Behring Diagnostics) was kept in small aliquots at −70°C in a stock solution of 1 mg/ml in dimethylsulphoxide (Grade 1, Sigma). An aliquot was thawed immediately before use.

Opsonised zymosan was prepared as previously described.²³ Zymosan (Sigma, type A) was boiled in water. After washing it was resuspended in HBSS at 20 mg/ml and stored at 4°C. Opsonised zymosan was prepared by incubating the stock zymosan with fresh human serum for 30 min at 37°C. It was then centrifuged and resuspended at 1 mg/ml in HBSS. N-formylmethionyl-leucyl-phenylalanine (FMLP) (Sigma) was stored in phosphate buffered saline (pH7) at a concentration of 2×10⁻³ M at 4°C.

**DETECTION OF RESPIRATORY BURST ACTIVITY**

Release of RMO by macrophages in response to the triggers was assessed by reduction of nitroblue tetrazolium (NBT; Sigma) to a deep blue-black formazan reaction product. Nitroblue tetrazolium was dissolved in RPMI at a concentration of 1 mg/ml and filtered with a 0.2 μ filter just before use.

1.5×10⁶ intestinal mononuclear cells in 4 ml culture medium were incubated at 37°C with 1 ml NBT solution (1 mg) and either PMA at a final concentration of 200 ng/ml, 75 μg opsonised zymosan (final concentration, 15 μg/ml) or FMLP at a final concentration of 10⁻² M. Cells stimulated with PMA and FMLP were incubated for 30 minutes and those with opsonised zymosan for 60 minutes. Controls included incubating the cells with culture medium and NBT only or with dimethylsulphoxide, NBT and culture medium. All the cell cultures were shaken at intervals of 15 minutes. Control experiments and those with triggers were performed concomitantly.

At the end of the incubation period, cyt centrifuge preparations (Cytospin 2; Shandon) were made, air dried and fixed in acetone. They were then stored at −20°C before staining.

Cyt centrifuge preparations of mononuclear cells incubated with PMA or FMLP were stained with monoclonal antibody Y1/82A which is specific for monocytes and macrophages²⁴²⁵ (kindly donated by Dr D Y Mason, John Radcliffe Hospital, Oxford). Preparations of mononuclear cells incubated with zymosan were stained with an anti-HLA-D monclonal antibody 2015Z which is specific for monocytes and macrophages²⁶ (kindly donated by Dr S Fuggle, John Radcliffe Hospital).

Staining of the cyt centrifuge preparations was performed using the peroxidase technique.²⁷ Endogenous peroxidase activity was blocked with methanol and hydrogen peroxide. Controls included omitting the primary antibody or using an irrelevant monoclonal antibody.
In cytocentrifuge preparations of cells incubated with opsonised zymosan, macrophages were considered to be those cells that were HLA-D positive and had phagocytosed the zymosan. For cells incubated with PMA or FMLP, macrophages were considered to be Y1/82A stained cells. Of these populations of macrophages, the percentage (in each cytocentrifuge preparation) showing release of RMO (as shown by blue-black formazan staining) was determined by counting at least 200 macrophages per preparation. All the cytocentrifuge preparations were coded and analyses were performed blind by one investigator (YRM). Results of many of these were confirmed by other investigators (variation less than 10%).

**IFN-γ and LPS Stimulation**

In some experiments, mononuclear cells isolated from normal or inflamed mucosa were stimulated with IFN-γ and LPS. The source of IFN-γ was supernatant from Chinese hamster ovary cell line (donated by Dr Scott, Wellcome biotech). Its activity was determined by immunoradiometric assay. It was used in a final concentration of 100–1000 U/ml. LPS was from *E coli* 055:B5 (Sigma) and was used at a concentration of 10 μg/ml.

The mononuclear cells (in suspension) were incubated with these stimuli for 42 hours. Respiratory burst activity by the macrophages, in response to PMA, was then determined as described above.

**Monocytes**

To study the effect of collagenase treatment on respiratory burst activity, peripheral blood monocytes were studied. Venous blood was collected in heparinised tubes and mononuclear cells were isolated by centrifugation on Ficoll-Paque (Pharmacia). 10⁶ mononuclear cells were shaken with 1 mg/ml collagenase (in culture medium) at 37°C for 3 hours. Monocytes adherent to glass coverslips were obtained from collagenase treated and untreated mononuclear cells by incubation at 37°C for 30 minutes. Non-adherent cells were removed by gentle washing with warm (37°C) 5% FCS/RPMI. Coverslips with adherent monocytes were incubated with culture medium, NBT (1 mg/ml) and PMA (at a final concentration of 200 ng/ml) at 37°C for 30 minutes. After washing, the coverslips were fixed in 0-1% gluteraldehyde for 15 minutes, washed and examined by phase contrast microscopy. The percentage of adherent cells undergoing respiratory burst (as shown by blue-black formazan staining) was determined.

**Statistical Analysis**

Statistical analysis was performed using Wilcoxon's rank-sum test (paired or unpaired as indicated). The analyses were two-tailed. As data were not normally distributed, results are expressed as a median and range.

**Results**

The viability of isolated intestinal and peripheral blood mononuclear cells was always 90% or more assessed by trypan blue exclusion.

Monocytes from three normal, healthy individuals were used to study the effect of collagenase treatment on respiratory burst activity. There was no significant difference in the proportion of collagenase treated or untreated adherent cells that were able to undergo respiratory burst (untreated – mean 82%; collagenase treated – mean 81%).

In cytoispin preparations of isolated intestinal mononuclear cells from normal and inflamed ileum and colon, there was no significant difference in the proportion of macrophages (identified with monoclonal antibody Y1/82A) present; normal colon: median 13-5% (range 7–22%); IBD colon median 12% (range 7–21%); normal ileum 12% (range 10–18%); ileal Crohn’s disease median 14% (range 10–19%).

**Nitroblue Tetrazolium Reduction by Isolated Intestinal Macrophages in the Absence of a Trigger**

Initially two controls were used. In one the cells were incubated with culture medium and NBT only for one hour and in the other, they were incubated with dimethylsulphoxide in addition to NBT and culture medium. After some experiments, however, no difference between these two controls were observed (data not shown). Therefore in subsequent experiments, only the control with dimethylsulphoxide was used.

In the absence of a trigger, there was a significant (p<0.05) increase in the proportion of macrophages isolated from inflamed colonic and ileal mucosa that reduced NBT compared with those isolated from mucosa of normal colon and terminal ileum (Fig. 1).

**Respiratory Burst Activity of Isolated Intestinal Macrophages in Response to Opsonised Zymosan**

Preliminary studies using varying doses and time intervals showed that 15 μg/ml of opsonised zymosan incubated for one hour gave optimal results for mononuclear cells obtained from both normal and inflamed mucosa.

In cytocentrifuge preparations, 75–90% of HLA-D positive macrophages (as assessed by their morphology) had phagocytosed zymosan. There was no difference between normal and inflamed intestine
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% of macrophages reducing NBT

Control colon (n=16) IBD colon (n=24) Control ileum (n=5) Ileal Crohn's (n=10)

Fig. 1 Percentage of macrophages reducing NBT in the absence of any trigger. Isolated intestinal mononuclear cells were incubated with NBT, culture medium and dimethylsulphoxide only. Cytocentrifuge preparations were then made and the proportion of macrophages (Y1/82A positive) reducing NBT was determined.

(data not given). These cells were easy to identify and of these, the percentage undergoing respiratory burst as shown by deep blue black staining was determined (Fig. 2).

Only a small proportion of macrophages isolated from normal colon were able to undergo a respiratory burst (median 8%; range 2–33%). A significantly greater proportion of macrophages isolated from inflamed colonic mucosa showed evidence of release of RMO (median 17%; range 9–49%; p<0.01) (Fig. 3).

Similarly, a significantly greater proportion of macrophages isolated from inflamed mucosa of ileal Crohn's disease showed evidence of having undergone a respiratory burst (median 22%; range 5–49%) compared to macrophages isolated from normal terminal ileal mucosa (median 9%; range 5–17%; p<0.05). There was no difference in the ability of macrophages isolated from different areas of normal colon to release RMO in response to opsonised zymosan (data not shown). There was also no difference between ulcerative colitis and Crohn's disease in the proportion of isolated colonic macrophages able to undergo a respiratory burst.

In six inflammatory bowel disease colons resected (four with ulcerative colitis and two with Crohn's disease), it was possible to study from the same colon, macrophages isolated from inflamed as well as from areas which were normal or near normal macroscopically and histologically. In five of six colons, fewer macrophages isolated from non or minimally inflamed mucosa showed evidence of release of RMO compared with those isolated from severely inflamed areas. In the sixth colon, no difference was seen (Fig. 4).
Macrophages were easy to identify with the monoclonal antibody Y1/82A (giving a brown reaction product) and the proportion releasing RMO (blue black staining) was determined. Studies using another macrophage specific monoclonal antibody 3C10 gave similar results to those obtained with Y1/82A (data not shown). The latter was chosen because of good and consistent staining using the peroxidase technique. In this system, contaminating polymorphs and eosinophils could be ignored as they did not stain with the monoclonal antibody (endogenous peroxidase activity having been blocked).

As was found for opsonized zymosan, only a small proportion of macrophages isolated from normal colon and ileum were able to undergo a respiratory burst (median 9%; range 1–45% and median 6%; range 4–11% respectively). There was a significantly higher proportion of macrophages isolated from mucosa of inflamed colon (p<0.01) and ileum (p<0.01) that showed evidence of release of RMO (median 45%; range 21–91% and median 43%; range 34–77% respectively) (Fig. 5). Macrophages from inflamed mucosa also showed more intense blue black staining suggesting a greater release of RMO per cell.

In five colons resected (three with ulcerative colitis and two with Crohn’s disease) macrophages isolated from severely inflamed and non or minimally inflamed areas of the same colon were studied (Fig. 6). In all five, a greater proportion of macrophages isolated from severely inflamed mucosa showed evidence of release of RMO compared with those from non or minimally inflamed mucosa.
RESPIRATORY BURST ACTIVITY OF ISOLATED INTESTINAL MACROPHAGES IN RESPONSE TO FMLP

Preliminary studies showed that $10^{-7}$ M of FMLP was optimal for triggering the respiratory burst (data not shown). N-formylmethionyl-leucyl-phenylalanine was not able to trigger macrophages isolated from normal colon and ileum to undergo a respiratory burst (Fig. 7). For macrophages isolated from inflamed ileal and colonic mucosa, FMLP was much less potent at triggering respiratory burst activity than opsonised zymosan or PMA. This was shown by a smaller proportion of cells isolated from inflamed mucosa demonstrating release of RMO as well as much less intense blue black staining per cell.

RESPIRATORY BURST ACTIVITY OF ISOLATED INTESTINAL MACROPHAGES AFTER STIMULATION WITH IFN-γ OR LPS

In preliminary studies, IFN-γ at a concentration of 680 U/ml was optimal for stimulating intestinal macrophages to undergo a respiratory burst. The viability of the mononuclear cells at the end of the incubation period (with IFN-γ or LPS) was always 80% or more.

Incubating the mononuclear cells in the presence or absence of IFN-γ or LPS did not influence the proportion of macrophage staining with Y1/82A (data not shown).

After incubation with IFN-γ there was a small increase in the proportion of macrophages from normal colonic mucosa that showed evidence of release of RMO (Table). Stimulation with LPS did not make any significant difference. In two experiments the mononuclear cells were incubated with IFN-γ and LPS, however the proportion of macrophages showing release of RMO was similar to that after incubation with IFN-γ alone. Incubating mononuclear cells isolated from inflamed mucosa with either IFN-γ or LPS did not produce any significant change in the proportion of macrophages undergoing a respiratory burst (Table).

**Discussion**

In the human ileum and colon macrophages are found in the lamina propria and are most prominent close to the basement membrane of the epithelium. Studies in our unit have shown morphological and immunohistochemical heterogeneity of these cells in normal and inflamed ileum and colon as well as in the granulomata of Crohn’s disease. There is little information on the functions of these intestinal cells.

This study investigated the capacity of isolated...
intestinal macrophages to undergo a respiratory burst as shown by the reduction of NBT to a deep blue black formazan reaction product in response to various triggers. This test gives a semiquantitative measure of respiratory burst activity in individual cells.\textsuperscript{16,17}

Superoxide anion, hydrogen peroxide and hydroxyl radicals can be more accurately determined quantitatively.\textsuperscript{9} These assays were not performed in this study because of difficulties in consistently excluding polymorphonuclear leucocyte contamination especially from mononuclear suspensions isolated from inflamed mucosa. In preliminary studies varying degrees of polymorphonuclear leucocyte contamination occurred despite centrifugation on Ficoll-Paque (performed twice) and separation of cells by adherence to fibronectin coated plates. This may reflect properties of young polymorphonuclear cells recruited into the mucosa. Any quantitative analysis of RMO in the presence of contaminating polymorphonuclear leucocytes would not accurately reflect production of these radicals by the macrophages.

The technique (NBT reduction) used in this study allows examination of most of the macrophage population rather than that selected – for example, by adherence or elutriation. In addition, this technique also enables study of the relationship of macrophage phenotype (as determined by monoclonal antibody or histochemical staining) and the functional capacity of the cell to release RMO. Our own and previous studies\textsuperscript{25} have shown that most of the human intestinal macrophages express MHC class II molecules. Our studies have also shown that most of these cells also stain with monoclonal antibody Y1/82A.\textsuperscript{25} Using two different ways to define the macrophage population and using different triggers, we have shown that a greater proportion of macrophages isolated from the mucosa of ulcerative colitis and Crohn's disease are able to undergo a respiratory burst compared with those isolated from normal terminal ileal or colonic mucosa.

During a respiratory burst, superoxide anion is formed by one-electron reduction of oxygen, a reaction catalysed by membrane-associated NADPH oxidase. In activated macrophages, this response is enhanced.\textsuperscript{7} Using this criteria, it appears that only a small proportion of normal intestinal macrophages are activated and that this proportion is increased in inflamed mucosa. As majority of human intestinal macrophages express HLA class II molecules,\textsuperscript{31} however it has been suggested that they are all activated.\textsuperscript{5} These differences may reflect heterogeneity in the activation of the macrophages. It is also possible that the intestinal macrophages express HLA-DR because they may be frequently required to present antigen to T Cells but are not activated as assessed by other functional tests.

It has been postulated that as monocytes migrate and mature into tissue macrophages, their ability to secrete large amounts of superoxide and hydrogen peroxide may be down regulated.\textsuperscript{19} This is supported by in vitro studies of superoxide anion and hydrogen peroxide production by monocytes after isolation and during differentiation into macrophages.\textsuperscript{11}

IFN-γ has been shown to activate macrophage oxidative metabolism.\textsuperscript{1} After stimulation with IFN-γ, one would have expected majority of the macrophages from normal colonic mucosa to be able to undergo a respiratory burst (in the presence of PMA). Only a small proportion were able to do so, however, suggesting that the resident population of intestinal macrophages in the normal colon are 'desensitised' to the effect of IFN-γ. The activity of this batch of IFN-γ had previously been determined and it has also been shown to induce HLA-DR in an epithelial cell line.\textsuperscript{12} The possibility that the 'desensitisation' to IFN-γ may be caused by the presence of tumour in the resected specimen cannot be excluded. One of the patients studied, however, had intestinal resection for idiopathic constipation, suggesting that this 'desensitisation' may be a phenomenon of normal intestinal macrophages.

This effect may be mediated by trace levels of bacterial lipopolysaccharide.\textsuperscript{33} In this respect, intestinal macrophages may have some similarities to murine Kupffer cells which fail to respond to IFN-γ \textit{in vitro} with a rise in their respiratory burst activity.\textsuperscript{10} Both may be exposed to trace levels of LPS which may cross the bowel wall into the portal circulation under physiological conditions.\textsuperscript{37} It is also possible that the lack of respiratory burst by these cells under normal conditions, protects from oxidative injury.\textsuperscript{36}

In active inflammatory bowel disease, there is infiltration by polymorphonuclear leucocytes, lymphocytes and macrophages.\textsuperscript{35} The greater proportion of intestinal macrophages from inflammatory bowel disease mucosa that are able to undergo a respiratory burst may be either the result of an upregulation of respiratory burst capacity of the resident population of cells or may reflect an elicited population of macrophages. It is generally believed that tissue macrophages in inflammatory lesions are predominantly derived from blood monocytes.\textsuperscript{31} Our studies with IFN-γ suggest that it is probably the elicited population of macrophages (derived from circulating monocytes) that contribute to the increased proportion able to release RMO.

Oxygen radicals produced by activated macrophages (as well as polymorphonuclear leucocytes) may be involved in mediating damage in the inflamed mucosa.\textsuperscript{4} The oxygen metabolites are likely to be
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