BASIC–ALIMENTARY TRACT

Microbial Mannan Inhibits Bacterial Killing by Macrophages: A Possible Pathogenic Mechanism for Crohn's Disease

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Background & Aims: **Crohn's disease (CD) is mimicked by inherited phagocyte disorders and is associated with circulating antibodies against yeast mannan (anti-*Saccharomyces cerevisiae* antibody; ASCA). We speculated that mannans might impair phagocyte function.** ***Methods:*** ***S cerevisiae* mannan was assessed for its effects on human peripheral blood neutrophils, adherent monocytes, and monocyte-derived macrophages (MDM).** ***Results:*** **Mannan caused dose-related increased survival of CD *Escherichia coli* HM605 within adherent monocytes from 24% ± 10.5% (control) to 114% ± 22.7% with mannan 1 mg/mL at 2 hours (mean ± SEM, n = 9; $P = .0002$). Electron microscopy showed *E coli* HM605 surviving and probably replicating within macrophage vesicles. Mannan (1 mg/mL) inhibited the respiratory burst in neutrophils and monocytes (both $P = .002$) and bacterial killing within MDM ($P < .001$). *E coli* survival was increased within macrophages from TLR4$^{-/-}$ (126% ± 3.5% survival at 2 hours) and MyD88$^{-/-}$ (134.8% ± 6.5%) mice compared with wild-type mice (both $P < .0001$). Mannan had no additional effect, showing that TLR4 and MyD88 are involved in bacterial killing by macrophages and its inhibition by mannan. Putative CD-associated micro-organisms were screened for the ASCA mannan epitope by *Galanthus nivalis* lectin (GNA) blotting. ASCA epitope was expressed by *Candida albicans* and *Mycobacterium paratuberculosis* but not by *Mycobacterium tuberculosis* or *E coli*. Supernatants from *M paratuberculosis* culture inhibited killing of *E coli* HM605 by adherent human monocytes and murine macrophages. The inhibitory activity was removed by GNA-affinity chromatography.** ***Conclusions:*** **Suppression of mucosal phagocyte function by microbial mannans, possibly of Mycobacterial origin, may contribute to CD pathogenesis.**

Crohn's disease (CD) is a poorly understood condition in which there is intestinal ulceration and inflammation that is characterized by the presence of granulomas containing coalescent macrophages.[1] The disease is complicated by sepsis, particularly by abscess and fistula formation, and bacteria have been grown from mesenteric lymph nodes[2,3] and identified by immunohistochemistry within macrophages in the mucosa.[4] There is no consistent finding of a single pathogen. *Mycobacterium paratuberculosis* can sometimes be found,[5–7] usually only by sensitive DNA detection[8,9] but occasionally by culture.[10–12]

Immunohistochemistry has shown *Escherichia coli*, *Listeria*, and *Streptococci* within macrophages in Crohn's tissue,[4] and *Escherichia coli* DNA has been found within Crohn's tissue granulomas.[13] Culture of mucosal biopsy specimens after removal of surface mucus has demonstrated increased numbers of *E coli* that differ from typical commensals by possessing the ability to adhere to and invade intestinal epithelial cell lines in culture.[14–17] A CD ileal *E coli* isolate has been shown to be able to replicate within phagolysosomes inside macrophages.[18] Patients with CD also commonly have circulating antibodies against bacterial flagellar antigens.[19–21]

All of this supports the hypothesis that CD may result from defective mucosal defense against the gut microbiota. Further support comes from identification of NOD2/CARD15 as a gene that is mutated in a significant minority of patients with CD.[22–24] This gene defect is associated with reduced killing of intracellular bacteria within transfected epithelial cells[25] and reduced production of bactericidal defensins by Paneth cells.[26] Moreover, intestinal disease that is arguably indistinguishable from CD occurs in 2 conditions in which there are well-characterized inherited defects in phagocyte function: chronic granulomatous disease[27] and glycogen storage disease

Abbreviations used in this paper: ASCA, anti-*Saccharomyces cerevisiae* antibody; CD, Crohn's disease; GNA, *Galanthus nivalis* agglutinin (lectin); LPS, lipopolysaccharide; Manα1–3Man, mannose α1–3 mannose; MDM, monocyte-derived macrophages; PMA, phorbol 12-myristate 13-acetate.

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type 1b.[28] Peripheral blood monocytes from patients with CD show a defective interleukin (IL)-8 response to peptidoglycan that is even more marked in individuals with abnormal NOD2. This is associated with defective neutrophil recruitment in keeping with defective phagocyte function as an underlying pathogenic mechanism.[29]

CD patients commonly have circulating antibodies to baker's yeast: anti-*Saccharomyces cerevisiae* antibody (ASCA).[30,31] The epitope for this antibody is a mannan with a specific mannose α1–3 mannose (Manα1–3Man) terminal disaccharide.[32,33] This is present in yeast cell walls, including *Candida albicans*,[34] but has also been shown to be expressed by transmembrane glycoproteins in *Mycobacterium bovis* and *Mycobacterium chelonae*.[35,36]

We speculated that mannan shed by intramucosal bacteria might inhibit phagocyte function and thus lead to impaired killing of intracellular bacteria within phagocytes and consequently to the granulomatous inflammation, abscess, and fistula formation that typify CD. It had previously been reported that yeast mannans can inhibit myeloperoxidase release,[37] and we now show that *S cerevisiae* mannan impairs a range of in vitro functions of normal human peripheral blood neutrophils, monocytes, and monocyte-derived macrophages (MDM). In particular, defective bacterial killing is demonstrated by adherent monocytes and MDM in the presence of mannans. This includes impaired killing of a CD mucosal adherent and invasive *E coli* isolate that shows inherent resistance to killing by macrophages.

Yeasts are relatively large organisms, which ought to be readily visible if present in CD tissue. Their apparent absence suggests an alternative source for the ASCA epitope. This epitope is selectively bound by the Snowdrop lectin, *Galanthus nivalis* agglutinin (GNA).[38] In lectin blotting studies, we have found that this lectin, as expected, recognizes yeast mannans not only in *S cerevisiae* but also in *Candida albicans* and also recognizes mannans in *Mycobacterium paratuberculosis* and *M bovis* but not in *Mycobacterium tuberculosis* or *E coli*. Supernatants from *M paratuberculosis* culture are shown to inhibit killing of CD *E coli* by human adherent monocytes and J774-A1 murine macrophages.

These studies support the hypothesis that CD may arise as a consequence of an acquired defect in phagocyte function, driven at least in part by cell wall mannans shed within the mucosa by gut-derived microbes. *M paratuberculosis* is a possible source for such mannans.

Materials and Methods

Reagents

Neutrophil isolation medium was obtained from Cardinal Associates Inc (Sante Fe, CA), and Ficoll-Hypaque was from Beckman Coulter, High Wycombe, United Kingdom. *S cerevisiae* yeast oligomannan (product code M3640), luminol (3-aminophthalhydrazide), isoluminol (4-aminophthalhydrazide), recombinant human tumor necrosis factor (TNF)-α, 5% pooled human AB serum, cytochrome c (horse heart), *E coli* lipopolysaccharide (LPS), horseradish peroxidase (HRP), phorbol 12-myristate 13-acetate (PMA), and formyl-Met-Leu-Phe were all obtained from Sigma (Poole, United Kingdom). Granulocyte Macrophage-Colony Stimulating Factor was from Roche Applied Science, Lewes, United Kingdom. Syto-24 green fluorescent nucleic acid stain and Mito-Tracker Red CMXRos were both obtained from Invitrogen Ltd, Paisley, United Kingdom. The mouse monoclonal anti-human IL-8 capture and detection antibodies (790A 28G2 and 893C 4G2) for the IL-8 enzyme-linked immunosorbent assay (ELISA) were purchased from Biosource, Camarillo, CA. Recombinant human IL-8 was purchased from Insight Biotechnology Ltd, Wembley, United Kingdom. Muramyl dipeptide (Ac-muramyl-Ala-D-Glu-NH_2) was obtained from Bachem Ltd, St. Helens, United Kingdom. Biotinylated GNA was purchased from Vector Labs, Peterborough, United Kingdom. GNA immobilized on cross-linked 4% beaded agarose was obtained from Sigma.

Isolation of Human Neutrophils, Adherent Monocytes, and MDM

Heparinized blood was obtained from consenting, healthy blood donors from the National Blood Transfusion Service. The study was approved by the Liverpool Local Research Ethics Committee. Neutrophils were isolated on neutrophil isolation medium, using the previously described method.[39] Contaminating erythrocytes were removed by hypotonic lysis with 0.1% NaCl. Purity and viability were assessed by May-Grünwald staining and trypan blue exclusion and were >95% and >97%, respectively. Purified neutrophils were suspended in RPMI 1640 medium or Hank's buffered salt solution (HBSS) containing 5.5 mmol/L D-glucose.

Human venous blood mononuclear cells were isolated from buffy coats by the Ficoll-Hypaque procedure.[39] Adherent monocytes were obtained by overnight culture on 12-well plastic culture plates in RPMI 1640 medium supplemented with 10% vol/vol fetal calf serum (FCS), 2% wt/vol L-glutamine, at 37°C in an atmosphere of 95% air, 5% CO_2. Nonadherent lymphocytes were removed by washing the culture wells with serum-free RPMI 1640 medium. MDM were obtained by further culture of adherent monocytes in complete RPMI media containing 50 U/mL Granulocyte Macrophage-Colony Stimulating Factor for 5–7 days. The murine macrophage-like cell line J774-A1 (ECACC 85011428) was used for electron microscopic studies and was obtained from the European Collection of Animal Cell Culture (Public Health Laboratory Service; Wiltshire, United Kingdom).

Bacterial Growth and Opsonization

CD mucosa-associated *E coli* (HM605, HM427, HM670, HM580, and HM95) were isolated as previously