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 man-nans, possibly of Mycobacterial origin, may contribute to CD pathogenesis.

C rohn’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 nodes2,3 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 detection8,9 but occasionally by culture.10–12 Immuno-histochemistry 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 cells25 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 disease27 and glycosgen 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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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 manno α1–3 manno (Manα1–3Man) terminal disaccharide.52,33 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-aminopphthalhydrazide), isoluminol (4-aminopphthalhydrazide), 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 Mitotracker 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 obtained from Insight Biotechnology Ltd, Wembley, United Kingdom. Muramyl dipeptide (Ac-muramyl-Ala-D-Glu-NH2) 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 fromuffy 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% CO2. 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
late cultures, were grown in biology terococcus faecalis cytogenes VA). American Type Culture Collection (ATCC; Manassas, Culosis. B2 or D, typical of CD mucosal isolates. All except HM95 (group A) belong to phylogenetic groups and replicate within I407 intestinal epithelial cells, and deionized water and vigorous vortexing for 1 minute. Neutrophils were then lysed by 2000-fold dilution in broth: 3 volumes of ice-cold sterile PBS and whirl mixing of 3 volumes of ice-cold sterile PBS and whirl mixing. Similarly, J774-A1 cells were sedimented overnight at 4°C and bacteria resuspended in RPMI 1640 medium. The culture supernatant was 0.2 mg/mL packed gel, washed in sterile PBS) per milliliter L GNA-agarose (3 mg sterile filtered to remove residual organisms. In addition, experiments were performed using M paratuberculosis culture supernatant following removal of Manα1–3Man-containing glycoconjugates by lectin-affinity “pull-down” using Snow-drop lectin GNA immobilized on cross-linked 4% beaded agarose. This lectin has been shown to have high specificity for the Manα1–3Man disaccharide epitope. “Pull-down” was performed for 2 hours at room temperature using 100 μL GNA-agarose (3 mg lectin/mL packed gel, washed in sterile PBS) per milliliter M paratuberculosis culture supernatant or control media.

Phagocytosis of Live, Opsonized Bacteria by Adherent Monocytes

S. aureus and the CD-associated E. coli HM605 were incubated in the dark with 1 μmol/L cell-permeating described. All these isolates had been shown to adhere to and replicate within 1407 intestinal epithelial cells, and all except HM95 (group A) belong to phylogenetic groups B2 or D, typical of CD mucosal isolates. E. coli ATCC 25922 (FDA strain Seattle 1946) was obtained from the American Type Culture Collection (ATCC; Manassas, VA). Staphylococcus aureus (Oxford strain), Listeria monocytogenes, Streptococcus bovis, Bacteroides fragilis, and Enterococcus faecalis were grown from stored isolates in the University of Liverpool, Division of Medical Microbiology. Whole live M. paratuberculosis, routine field isolate cultures, were grown in para-JEM liquid culture broth (TREK Diagnostic Systems Ltd, East Grinstead, United Kingdom) containing a nominal volume of 10 mL of a modified Middlebrook 7H9 broth and compressed sponges. At the point of sample inoculation, a total of 2.5 mL of the following 3 supplements was also added to the broth: para-JEM GS (growth supplement), para-JEM AS (antibiotic supplement), and para-JEM EYS (egg yolk supplement). Culture was for 56 days at 37°C. M. paratuberculosis was also positive for growth at approximately 8 weeks on Euden’s 7H11 solid agar containing Mycobactin J. A suspension in phosphate-buffered saline (PBS), pH 7.6, was made to a turbidity approximating McFarlands 1.0 (equating to 1 × 10^8 organisms/mL).

Heat-inactivated M. paratuberculosis (7 isolates), M. tuberculosis, M. avium, M. kansasii, and M. chelonae were kind gifts from Professor Glyn Hewinson; The Veterinary Laboratories Agency, Addlestone, Surrey, United Kingdom. All bacteria were grown overnight on nutrient agar plates at 37°C, harvested, and resuspended in sterile PBS, pH 7.4. Bacteria were quantified by optical density (OD) at 600 nm using previously established calibration curves. As required, opsonization was performed by incubating bacteria (5 × 10^7/mL) with 10% vol/vol heat-inactivated pooled human serum from healthy donors for 30 minutes at 37°C before addition of 3 volumes of ice-cold sterile PBS and whirl mixing for 30 seconds. Subsequently, bacteria were centrifuged at 1000g for 15 minutes, washed, and recentrifuged 3 times in sterile PBS. Bacteria were finally resuspended in sterile PBS at experimental working concentrations. Heat inactivation of bacteria was performed at 60°C for 30 minutes.

Killing of Bacteria by Human Neutrophils, Suspended Mononuclear Cells, Adherent Monocytes, and MDM

Using freshly isolated neutrophils, killing of opsonized bacteria was examined in cells after pretreatment with 0, 0.125, 0.25, 0.5, and 1.0 mg/mL S. cerevisiae mannan in RPMI 1640 medium for 30 minutes. Opsonized bacteria were mixed with neutrophils at a ratio of 10:1 followed by gentle agitation for 30 minutes at 37°C. Neutrophils were then lysed by 2000-fold dilution in deionized water and vigorous vortexing for 1 minute. Aliquots were spread plated onto nutrient agar and colony-forming units (cfu) counted after overnight incubation. Bacterial killing was expressed as the percentage of bacteria surviving in mannan-treated cells compared with untreated controls.

Suspended mononuclear cells, adherent monocytes, and MDM, (all at 1 × 10^6 per mL) in RPMI medium supplemented with 10% vol/vol FCS, 2% wt/vol L-glutamine, and HEPES, were gently agitated in a 37°C incubator in the presence or absence of 1 mg/mL S. cerevisiae mannan for 1 hour. Cells were then incubated with 1 × 10^6 S. aureus or E. coli HM605 at a 1:1 bacterium to cell ratio. For adherent monocytes, a dose response assay was also performed to assess the effect of mannan concentrations of 0, 0.125, 0.25, 0.5, and 1.0 mg/mL on the killing of CD mucosa-associated E. coli HM605. The effect of mannan on bacterial killing by adherent monocytes was also assessed for a further 4 CD mucosa-associated E. coli strains: HM427, HM670, HM580, and HM95. After 0.5 and 2 hours, wells were washed 3 times with warm (37°C) PBS before lysis of adherent cells with 1% vol/vol Triton X-100. An aliquot of each lysate was plated on nutrient agar plates, in triplicate, and incubated at 37°C for 24 hours, and numbers of cell-associated bacteria were determined by counting of cfu. In adherent cells, bacterial killing was expressed as the percentage of bacteria surviving at T 2 hours compared with T 0.5 hours. In suspended cells, data were expressed as the percentage of bacteria surviving at T 2 hours compared with T 0 hours.

The effect of M. paratuberculosis whole live bacteria and culture supernatants was assessed on killing of CD E coli HM605 by adherent human monocytes (as above) as well as on J774-A1 murine macrophages. J774-A1 cells were seeded into 12-well tissue culture plates at 1 × 10^4 cells per well, maintained in RPMI 1640 medium, and used 24 hours later. Whole live M. paratuberculosis (10^7/mL) were sedimented overnight at 4°C and bacteria resuspended in RPMI 1640 medium. The M. paratuberculosis culture supernatant was 0.2 μm sterile filtered to remove residual organisms. In addition, experiments were performed using M. paratuberculosis culture supernatant following removal of Manα1–3Man-containing glycoconjugates by lectin-affinity “pull-down” using Snow-drop lectin GNA immobilized on cross-linked 4% beaded agarose. This lectin has been shown to have high specificity for the Manα1–3Man disaccharide epitope. “Pull-down” was performed for 2 hours at room temperature using 100 μL GNA-agarose (3 mg lectin/mL packed gel, washed in sterile PBS) per milliliter M. paratuberculosis culture supernatant or control media.
Syto-24 at room temperature for 15 minutes. Bacteria were washed once and resuspended in sterile PBS. Fluorescent bacteria were added, at a ratio of 10:1, to adherent monocytes, which had been labelled with 25 μmol/L MitoTracker Red and preincubated in the dark for 30 minutes in the presence or absence of 1 mg/mL mannan. Cell-associated fluorescence, following 30 minutes incubation, was visualized using an LSM510 confocal microscope (Carl Zeiss, New York, NY) at 1024 × 1024 pixel resolution through a ×63 Plan Apochromat (na 1.4) objective with 2 times averaging. Fluorescence emission was collected through a 505–550 nm band-pass filter (green Syto 24).

**Phagocytosis of Heat-Killed, Opsonized Bacteria by Neutrophils**

Phagocytosis of heat-killed bacteria was assessed as previously described.38 Heat-killed *S aureus* were resuspended in sterile PBS containing 30 μmol/L propidium iodide. The suspension was incubated in the dark at 4°C for 2 hours. Labelled bacteria were washed 3 times in sterile HBSS containing 0.1% wt/vol gelatin and then opsonized as described above. Opsonized bacteria were then incubated with neutrophils at a ratio of 10:1. Experiments were performed using neutrophils preincubated in the presence or absence of 1 mg/mL *S cerevisiae* mannan, with gentle agitation in the dark for 30 minutes, at 37°C. After treatment with bacteria, the neutrophils were pelleted by centrifugation, washed twice, and then fixed in PBS containing 5 mmol/L EDTA, 3 mmol/L sodium azide, and 1% paraformaldehyde. Cells were analyzed immediately by flow cytometry, and red fluorescence was collected through a 620-nm long pass filter. Ten thousand gated events were collected in the neutrophil gate for each sample.

**Transmission Electron Microscopy of Macrophages**

Subsequent to infection, as described above, J774-A1 murine macrophages were fixed in 2% glutaraldehyde and 4% paraformaldehyde in PBS. Cells were then washed in PBS (5 minutes), twice in PBS–0.15 mol/L glycine (2 × 5 minutes), and then 1 mL PBS was added per plate. Cells were carefully scraped into microfuge tubes containing PBS, centrifuged at 9000g for 2 minutes, 2% agarose added, centrifuged at 9000g for 2 minutes, and then placed on ice until the agarose set. Samples were trimmed to 1-mm³ pieces, incubated in 1% osmium tetroxide for 1 hour at room temperature, followed by sequential dehydration in ethanol and acetone, and mounted in araldite resin. Seventy-nanometer-thick sections were loaded onto copper grids, stained for 5 minutes each in Reynolds lead citrate and 5% uranyl acetate, washed for 1 minute in distilled water, air-dried for 24 hours, and examined using an FEI 120 kV Tecnai G2 Spirit BioTWIN transmission electron microscope (FEI Company, Hillsboro, OR).

**Killing of Bacteria in Murine Bone Marrow-Derived MyD88−/− and TLR4−/− Macrophages**

Mice deficient in MyD88 and TLR4 (on a C57Bl/6 background) were kindly made available to Dr Stuart Marshall-Clarke by Prof. R. K. Gencris (School of Biological Sciences, University of Manchester, United Kingdom), with the generous permission of Prof. S. Akira (Osaka University, Japan).50 C57Bl/6 wild-type mice were purchased from B & K Universal (North Humberside, United Kingdom) and maintained in the animal facility at University of Liverpool. Mice were studied at >8 weeks of age. All animal studies were carried out in accordance with United Kingdom Home Office regulations for animal care and use.

For these experiments, 3 control C57Bl/6, 3 TLR4−/−, and 2 MyD88−/− mice were used. All experiments were conducted in triplicate. To generate macrophages, bone marrow cells harvested from the femurs of recently killed C57Bl/6, TLR4−/−, and MyD88−/− mice were cultured at 5 × 10⁵ cells per milliliter in RPMI 1640 medium containing 10% vol/vol FCS and 20% vol/vol murine macrophage colony stimulating factor-containing medium derived from mouse lymphoblast cells (LADMAC; ATCC, Manassas, VA). After 6 days, adherent cells were harvested and washed twice in RPMI media. Macrophages were then resuspended in RPMI 1640 medium, supplemented with 10% vol/vol FCS, and 2% wt/vol L-glutamine, and cultured overnight in 24-well plates at 100,000 cells/250 μL per well at 37°C. Medium, with or without 1 mg/mL *S cerevisiae* mannan, was replaced, and cells were incubated for a further 1 hour. Infection with opsonized *E coli* ATCC 25922 or CD mucosa-associated *E coli* HM605 was performed at cell:cell ratio 1:1. Again, bacterial killing was expressed as the percentage of bacteria surviving at T 2 hours compared with T 0.5 h.

**Measurement of Neutrophil and Peripheral Blood Monocyte Reactive Oxygen Metabolites During the Respiratory Burst**

The generation of reactive oxidants was measured by luminol- and isoluminol chemiluminescence. For neutrophils, 10⁶ cells were incubated in HBSS at 37°C (total volume of 1 mL) containing either 10 μmol/L luminol or isoluminol (plus 1 μmol/L HRP) to measure intracellular and extracellular chemiluminescence, respectively.29 Cells were stimulated by the addition of 0.1 μg/mL PMA (monocytes) or 1 μmol/L formyl-Met-Leu-Phe (neutrophils). For peripheral blood monocytes, isoluminol-dependent chemiluminescence was measured using a Bio-Rad 3550 kinetic plate reader (Bio-Rad, Hemel Hempstead, United Kingdom) in wells containing cell suspensions of 200 μL. For measurements of O₂− secretion, neutrophils were suspended at 5 × 10⁵ cells/mL in HBSS supplemented with 75 μmol/L cytochrome c as described previously.39 Following addition of
PMA (0.1 μg/mL), absorption increases, representing superoxide dismutase (SOD)-inhibitable O₂⁻ production, were monitored at 550 nm. HOCl production was measured by the formation of taurine chloramine.39 S cerevisiae mannan (0–1 mg/mL) was added to neutrophils or peripheral blood monocytes (1 × 10⁶ cells/mL) in HBSS or RPMI 1640 medium containing 20 mmol/L HEPES. Samples were preincubated for 5 minutes at 37°C prior to measuring reactive oxygen metabolite production after addition of 0.1 μg/mL PMA or heat-killed serum-opsonized S aureus (1 × 10⁷ bacteria/mL) to stimulate the respiratory burst.

**Effect of S cerevisiae Mannan on IL-8 Release From Human MDM in Response to Muramyl Dipeptide**

MDM (5 × 10⁶) were incubated, in triplicate, for 1 hour in the presence or absence of 1 mg/mL S cerevisiae mannan. Cells were then treated with 100 ng/mL muramyl dipeptide for 4 hours. Following treatment, IL-8 released to the medium was measured in triplicate using a solid-phase sandwich ELISA with anti-human IL-8 capture and detection antibodies.

**Identification of the Manα1–3Man Epitope in Bacteria and Yeast**

Bacteria examined included heat-inactivated M paratuberculosis (7 isolates), M tuberculosis, M avium, M kansasii, M bovis, and M cheloneia. Staphylococcus aureus (Oxford strain), Streptococcus bovis, B fragilis, E faecalis, L monocytogenes, CD mucosa-associated E coli isolate HM427, S cerevisiae and C albicans yeast cultures were also included. For each isolate, 5 × 10⁶ bacteria counts determined by OD 600 nm, or 1 × 10⁶ of yeasts, suspended in 200 μL sterile PBS, pH 7.4, were slot blotted under vacuum onto a nitrocellulose membrane. S cerevisiae mannan (200 μg) was used as a positive control. Protein loading was assessed using 0.1% wt/vol Ponceau S in 5% vol/vol acetic acid. Following a brief wash in 0.2% acetic acid, membranes were fixed with 10:25:65 vol/vol/vol acetic acid:isopropyl alcohol:water and then reequilibrated in sterile PBS. Unbound membrane was then blocked with 1% wt/vol bovine serum albumin in PBS overnight at 4°C, followed by incubation with 0.2 μg/mL biotinylated Snowdrop lectin GNA in PBS containing 1% wt/vol bovine serum albumin and 0.1% vol/vol Tween for 1 hour at room temperature. GNA shows high specificity for the Manα1–3Man disaccharide epitope38 and has previously been used to identify this epitope in M cheloneia, with confirmation of oligosaccharide structure by spectroscopic analysis.36 Lectin binding was amplified using ExtrAvidin (Sigma, Poole, UK) peroxidase conjugate (1:10,000 dilution), followed by chemiluminescence detection with SuperSignal West Dura Extended Duration Substrate (Perbio Science, Cramlington, United Kingdom) and visualization using Quantity One software (Bio-Rad).

**Statistics**

Statistical analysis was performed, unless otherwise stated, using 1-way ANOVA followed by selected pairwise comparisons of treatment means with the Bonferroni modified t test (2-tailed). Differences were considered significant when P < .05.

**Results**

**Inhibitory Effect of Yeast Mannan on Bacterial Killing by Adherent Human Monocytes**

**Killing of E coli.** The presence of S cerevisiae mannan in the culture medium caused a dose-related increase in survival of the CD E coli isolate HM605 within adherent human monocytes from 24% ± 10.5% in the absence of mannan to 114% ± 22.7% in the presence of mannan 1 mg/mL (mean ± SEM) at 2 hours (n = 9; P = .0002, Figure 1A) (in the Results section, n = number of experiments in all cases). The CD E coli isolate HM605 was much better able than the control E coli ATCC 25922 to survive within adherent human monocytes, showing 36% median survival at 2 hours (range, 6%–57%) compared with 1% (range, 0%–4%; P = .03) survival for E coli ATCC 25922 (Figure 1B). Electron microscopy showed that E coli HM605 was incorporated into J774 murine macrophage vesicles, with some bacteria appearing to replicate within vesicles (Figure 1C), as reported for a CD ileal E coli isolate.18 The 4 other adherent and invasive CD-associated E coli isolates tested (HM427, HM670, HM580, and HM95) also showed increased survival (42.5% median survival) within adherent human monocytes at 2 hours when compared with E coli ATCC 25922 (P = .001 by Mann–Whitney U test). In each case, preincubation with mannan substantially increased percent-age survival of the CD-associated E coli at 2 hours, whereas mannan (1 mg/mL) had a modest effect (P = .06) on the killing of E coli ATCC 25922 (Table 1).

**Killing of S aureus.** The presence of mannan also suppressed killing of S aureus by adherent monocytes, but this effect only approached significance by 2 hours (n = 6; P = .07, Figure 1B).

**Inhibitory Effect of Yeast Mannan on Bacterial Killing by Adherent Human MDM**

**E coli.** The presence of mannan at 1 mg/mL, the concentration shown to inhibit bacterial killing within adherent monocytes, also increased survival of E coli HM605 within adherent MDM from 72.5% ± 11% (mean ± SEM) in control MDM at 2 hours to 210.2% ± 22.5% survival (n = 6; P < .001, Figure 2).

**S aureus.** Again, mannan had a relatively modest and nonsignificant effect on the survival of S aureus within adherent MDM (P = .18, Figure 2).
Lack of Inhibitory Effect of Yeast Mannan on Bacterial Killing by Suspended Human Mononuclear Cells

Mannan treatment (1 mg/mL) had no significant effect on the killing of CD-associated *E. coli* or *S. aureus* by suspended mononuclear cells (Figure 3).

Mannan Does Not Interfere With Phagocytosis of Opsonized Bacteria

Mannan did not interfere with phagocytosis of opsonized live bacteria by adherent MDM as assessed by both confocal microscopy and flow cytometry (total cell-associated fluorescence: 49,604 ± 568 in untreated cells).

### Table 1. Effect of Mannan Coincubation on Survival of Various Crohn’s Disease and Control *E. coli* Within Adherent Monocytes at 2 Hours

<table>
<thead>
<tr>
<th><em>E. coli</em> Source</th>
<th>N</th>
<th>Percentage survival without mannana</th>
<th>Percentage survival with mannana</th>
<th>P value (Mann–Whitney U test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC 25922</td>
<td>6</td>
<td>3.8 ± 2.7</td>
<td>10.8 ± 3.5</td>
<td>.06</td>
</tr>
<tr>
<td>CD HM605</td>
<td>10</td>
<td>46.1 ± 9.1</td>
<td>140.5 ± 35.8</td>
<td>.004</td>
</tr>
<tr>
<td>CD HM427</td>
<td>2</td>
<td>411.0 ± 38.2</td>
<td>1187.5 ± 224.5</td>
<td></td>
</tr>
<tr>
<td>CD HM670</td>
<td>1</td>
<td>28.1 ± 5.1</td>
<td>51.8 ± 6.8</td>
<td></td>
</tr>
<tr>
<td>CD HM580</td>
<td>1</td>
<td>18.0 ± 5.1</td>
<td>56.7 ± 6.8</td>
<td></td>
</tr>
<tr>
<td>CD HM95</td>
<td>1</td>
<td>26.3 ± 5.1</td>
<td>78.5 ± 6.8</td>
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</tr>
</tbody>
</table>

**NOTE.** *P* < .0001 for comparison across all paired mannan-treated/untreated samples using Wilcoxon signed ranks test.
infected with Syto24-labelled *E. coli* HM605 compared with 49,301 ± 539 in 1 mg/mL mannan-treated cells). Similar results were obtained for Syto24-labelled opsonized live *S. aureus*. In addition, no difference was observed in the phagocytosis of heat-killed, propidium iodide-labelled, serum-opsonized *S. aureus* in the presence or absence of mannan as assessed by confocal microscopy: normalized values were control, 100% (±7%, n = 11), and mannan treated, 105% (±12%, n = 15, NS).

**Killing of Bacteria by Murine Bone Marrow-Derived MyD88<sup>−/−</sup> and TLR4<sup>−/−</sup> Macrophages**

Bone marrow-derived macrophages from wild-type C57Bl/6 mice were able to kill both *E. coli* ATCC 25922 and the CD-associated *E. coli* HM605 to a similar level (ATCC 25922, 54% ± 12.1% and HM605, 53.1% ± 5.8% surviving after 2 hours; mean ± SD, Figure 4). *E. coli* HM605 survival was increased in macrophages derived from both TLR4<sup>−/−</sup> (126% ± 3.5%; P = .0001) and MyD88<sup>−/−</sup> (134.8% ± 6.5%; P = .0001) mice, resulting in net replication when compared with wild-type macrophage killing. Incubation with 1 mg/mL *S. cerevisiae* mannan enhanced survival of the CD mucosa-associated *E. coli* HM605 in wild-type C57Bl/6 macrophages to 111.6% ± 6.6% (P = .0006, n = 3) but had no significant further effect on the increased levels of survival already observed in either the TLR4<sup>−/−</sup> or the MyD88<sup>−/−</sup> macrophages (Figure 4). Similar results were seen using *E. coli* ATCC 25922, again with mannan significantly enhancing bacterial survival in wild-type C57Bl/6 macrophages (P = .0005) but with no additional effect of mannan on the already increased survival seen in the macrophages derived from MyD88<sup>−/−</sup> and TLR4<sup>−/−</sup> mice.

**Lack of Inhibitory Effect of Yeast Mannan on Bacterial Killing by Suspended Human Neutrophils**

Untreated suspended neutrophils were able to kill 52% ± 14%, n = 6 experiments (mean ± SEM), of *S. aureus* at 30 minutes. The presence of mannan had no significant effect on the killing of *S. aureus* at any concentration tested (n = 2, each tested in triplicate at 0.125, 0.25, 0.50, and 1 mg/mL mannan). At 1 mg/mL, killing by mannan-
treated cells was 122% ± 9.8% of control untreated cells ($P = .25$), and, at 0.25 mg/mL, killing was 125% ± 12.2% ($P = .27$).

**Inhibition of the Respiratory Burst by Mannan**

**Monocytes.** In control peripheral blood monocytes, peak respiratory burst activity was reached 12 minutes after stimulation by PMA. Mannan caused dose-dependent inhibition of PMA-stimulated chemiluminescence with 87.7% ± 1.3% inhibition at 1 mg/mL ($P = .002$, Figure 5A and B).

**Neutrophils.** *S cerevisiae* mannan also caused dose-dependent inhibition of the neutrophil respiratory burst (measured by luminol-dependent chemiluminescence) with 66% ± 4.2% (mean ± SEM) inhibition at 1 mg/mL ($P = .0002$, Figure 5C). In addition to inhibiting luminol chemiluminescence, which measures the combined activities of the NADPH oxidase and myeloperoxidase, mannan (1 mg/mL) also inhibited isoluminol-dependent chemiluminescence (by 58% ± 2.3%, $P = .03$) and cytochrome c reduction (by 39% ± 4.9%, $P < .0001$). Because both of the latter are measures solely of NADPH oxidase activity, these data indicate that mannan inhibits the activity of the NADPH oxidase rather than myeloperoxidase. This was confirmed in experiments showing that mannan had no inhibitory effect on myeloperoxidase activity in a cell-free system (data not shown). We also experimentally confirmed that mannan was not merely acting as a scavenger of reactive oxidants because it did not quench HOCl (in a taurine chloramine assay) nor did it quench O$_2^-$ or H$_2$O$_2$ in a xanthine/xanthine oxidase cell-free system (data not shown).

Mannan inhibited the neutrophil release of HOCl (a product of the respiratory burst). In the absence of neutrophil stimulation, there was no significant difference in HOCl secretion in untreated (21.6 ± 0.3 μmol/L) and mannan-treated neutrophils; (18.1 ± 0.02 μmol/L and 20.2 ± 0.02 μmol/L for 0.5 mg/mL and 1.0 mg/mL mannan, respectively). Following stimulation with PMA, HOCl secretion by control cells increased to 275 ± 0.54 μmol/L, whereas there was a decreased response in the mannan-treated neutrophils (71.5 ± 0.38 μmol/L and 58.8 ± 0.30 μmol/L, at 1 mg/mL and 0.5 mg/mL mannan, respectively (both $P < .0001$). In control experiments, mannan did not react directly with HOCl.

**Figure 5.** (A) Mannan dose dependently inhibits the peak respiratory burst in PMA prestimulated monocytes (n = 10; amplified by HRP and detected with isoluminol, $P = .0023$, Cuzick’s test for linear trend across the dose response (B)) Time course of inhibition of peak respiratory burst in PMA prestimulated monocytes by mannan (1 mg/mL), amplified by HRP and detected with isoluminol. Representative example performed with 10 replicates at each sample time point. (C) Mannan inhibits peak respiratory burst in formyl-Met-Leu-Phe prestimulated neutrophils in a dose-dependent manner (n = 4 or 5; $P = .0002$, Cuzick’s test for linear trend across the dose response).

**Effect of S Cerevisiae Mannan on IL-8 Release From Human MDM in Response to Muramyl Dipeptide**

MDM from CD patients with the NOD2/CARD15 mutation have been shown to have a defective IL-8 response to the NOD2 ligand, muramyl dipeptide, and even CD patients without the NOD2 defect had a lesser but significant defect in IL-8 response. The hypothesis was tested that *S cerevisiae* mannan might suppress the IL-8 response of MDM to muramyl dipeptide. This was not found to be the case. IL-8 release over 4 hours from human normal blood donor MDM increased from baseline 378.9 ± 105.7 to 921.0 ± 34.2 pg IL-8/mL after incubation with 100 ng/mL muramyl dipeptide ($P < .0001$) and to a similar extent (962.4 ± 39.9 pg IL-8/mL) when incubated with a combination of muramyl dipeptide (100 ng/mL) together with *S cerevisiae* mannan (1 mg/mL). There was also no suppression of the IL-8 response to the CD *E coli* HM605 (813.8 ± 127.8 pg/mL) in the presence of 1 mg/mL mannan (944.4 ± 90.7 pg/mL IL-8). A similar increase in IL-8 release was also seen in response to 1 mg/mL *S cerevisiae* mannan alone (938.8 ± 102.4 pg/mL IL-8).
Figure 6. Slot blotting probed with biotin-conjugated Snowdrop (Galanthus nivalis) lectin (GNA) recognizes terminal Manα1–3Man-expressing glycoconjugates on M. paratuberculosis, M. avium, M. kansasii, M. bovis, S. cerevisiae, and C. albicans. Crohn’s disease mucosa-associated E. coli HM427, M. tuberculosis, B. fragilis, E. faecalis, S. bovis, and L. monocytogenes were all negative. Optical density (OD; 600 nm) was used to measure bacterial cell number prior to loading of 5 x 10^6 bacteria or 1 x 10^5 yeasts per slot. S. cerevisiae mannan (200 μg) was used as a positive control and sterile PBS as a blank (background) control. Blots shown are representative of 3 replicates.

The Manα1–3Man ASCA Epitope Is Expressed by Mycobacterium avium var. paratuberculosis

All 7 isolates of M. paratuberculosis tested were shown to express the Manα1–3Man terminal oligosaccharide as demonstrated by binding of GNA (Figure 6). M. avium also strongly expressed Manα1–3Man with other Mycobacterium species demonstrating lower degrees of reactivity to GNA (M. kansasii > M. bovis > M. chelonae > M. tuberculosis). B. fragilis, S. bovis, E. faecalis, and E. coli isolate HM427 all showed no significant GNA reactivity. As expected, both yeast cultures tested, S. cerevisiae and C. albicans, strongly expressed the Manα1–3Man terminal oligosaccharide; n = 3 replicates for each.

Culture Supernatants From M. paratuberculosis but not Whole Live M. paratuberculosis Inhibit Killing of CD Mucosal E. coli HM605 by Human Adherent Monocytes and J774-A1 Murine Macrophages

M. paratuberculosis culture supernatant (0.2 μm sterile filtered) from 10^7 bacteria/mL in para-JEM liquid medium at 1:2 dilution (with RPMI 1640) increased survival of CD mucosal E. coli HM605 in human adherent monocytes (Figure 7A). At 2 hours, counts of bacteria released from adherent monocytes were 202.2 ± 137.28 cfu/well (mean ± SD) in the presence of M. paratuberculosis supernatant compared with almost complete killing (3.33 ± 7.79 cfu/well) in the presence of 1:2 control (ie, M. paratuberculosis negative) culture medium (P < .0001). Whole live M. paratuberculosis showed no significant effect on killing of E. coli HM605 by adherent human monocytes when tested at M. paratuberculosis/adherent monocyte ratios of 4:1 and 8:1. Two isolates were assessed: the first from liquid broth culture, as used in the previous supernatant experiments, E. coli survival: control, 11.73% ± 20.12% at 2 hours; M. paratuberculosis: monocyte 4:1 ratio, 10.29% ± 13.17%; 8:1 ratio, 9.92% ± 12.90%, n = 9); second isolate from solid agar culture: E. coli survival: control, 6.27% ± 4.68%; M. paratuberculosis: monocyte 4:1 ratio, 17.87% ± 18.32%; 8:1 ratio, 8.20% ± 4.54%, n = 9). In each experiment, S. cerevisiae mannan (1 mg/mL) again significantly increased survival of E. coli HM605 (P < .0001, n = 9).

M. paratuberculosis culture supernatant from 10^7 bacteria/mL in para-JEM liquid medium at 1:2 dilution increased survival of CD mucosal E. coli HM605 in J774-A1 murine macrophages from 43.04% ± 4.75% control (1:2 culture medium) to 120.95% ± 22.24% at 2 hours (n = 9, P < .0001) (Figure 7B); a separate dose-response study...
showed no significant inhibitory effect of 1:4 diluted *M. paratuberculosis* culture supernatant (data not shown). The inhibitory effect of the *M. paratuberculosis* culture supernatant (at 1:2 dilution) on killing of CD mucosal *E. coli* isolate HM605 in J774-A1 murine macrophages disappeared after removal of Manα1-3Man containing glycoconjugates by lectin-affinity “pull-down” using agarose-immobilized GNA (Figure 7B).

**Discussion**

These studies show that *S. cerevisiae* mannan impairs the ability of adherent monocytes and monocyte-derived macrophages to kill phagocyted *E. coli* and, to a lesser extent, *S. aureus*, suggesting that this is not a specific effect against killing of *E. coli*. The CD adherent and invasive *E. coli* isolate HM605 is shown to survive more readily within macrophages than the laboratory *E. coli* ATCC 25922, originally a urinary tract isolate. Both these *E. coli* isolates grew readily within macrophages derived from knockout TLR4−/− and MyD88−/− mice suggesting that TLR4 and downstream MyD88 are involved in bacterial killing by macrophages.

It is interesting to note that, whereas mannan inhibited the respiratory burst of both neutrophils and suspended monocytes, it did not significantly affect bacterial killing by these cell types but did inhibit bacterial killing by adherent monocytes and MDM. There are several possible explanations for this. Although mannan greatly decreased reactive oxidant production by neutrophils and suspended monocytes, inhibition was not complete, and the low residual NADPH oxidase activity that escaped inhibition may be sufficient to effect killing. This situation is reminiscent of chronic granulomatous disease in which patients may have a marked dysfunction of their NADPH oxidase and yet have little defect in their killing mechanisms. Alternatively, it may be that mannan also inhibits another key process that regulates bacterial killing by macrophages. This is supported by the fact that monocyte-derived human macrophages have greatly decreased NADPH oxidase activity and so must utilize nonoxidative killing mechanisms during phagocytosis. The killing of *E. coli* by neutrophils is, for example, known to occur efficiently via nonoxidative killing mechanisms such as neutrophil elastase.

*S. cerevisiae* mannan has previously been shown to induce TNF-α production by adherent monocytes via a CD14- and TLR4-dependent pathway. This effect required the presence of LPS binding protein but was not inhibited by polymixin B so was not dependent on LPS itself. The lack of impact of *S. cerevisiae* mannan on bacterial survival in TLR4−/− and MyD88−/− macrophages suggests that these pathways are also involved in the mannan-induced inhibition of bacterial killing by the macrophages. It is notable that the presence of mannan suppressed killing by control C57Bl/6 murine macrophages down to the level seen with TLR4−/− and MyD88−/− macrophages.

The demonstration that the ASCA epitope Manα1-3Man is expressed in *M. paratuberculosis* is intriguing. It has been proposed that *M. paratuberculosis* is present in CD tissue in a cell wall-deficient form, but it is important to note that in other mycobacteria the Manα1-3Man structure is expressed on a transmembrane glycoprotein that will still be present in cell wall-deficient organisms. The present study confirms the recently described expression of the ASCA epitope by *C. albicans*, but, if *C. albicans* is the source of the ASCA reaction in CD, it is surprising that there is no microscopic evidence of invasive candidosis. It has recently been reported that CD sera also contain antibodies against a range of other oligosaccharide epitopes including laminaribiose and chitobiose that might also be of microbial origin.

When Dalziel first described chronic enteritis in humans in 1913, he proposed that the disease was caused by the same organisms as those already known to be responsible for chronic enteritis, Johnne’s disease. It is remarkable that the causal role for the Johne’s disease organism, *M. paratuberculosis*, in CD is still uncertain nearly a century later. It seems reasonably clearly established that low counts of *M. paratuberculosis* can be identified within, and occasionally cultured from, CD tissue. The impressive therapeutic response of CD to the TNF-α antibody, infliximab, which is known to cause reactivation of tuberculosis, has led to increased scepticism that *M. paratuberculosis* could play any role in pathogenesis. The data presented in the current study do, however, support the possibility that *M. paratuberculosis*, although not itself a very effective pathogen in humans, might induce a local suppression of phagocyte function in infected tissues and that this might in turn lead to chronic replication within macrophages of other bacteria, perhaps particularly the mucosal *E. coli* isolates that have now been found in CD tissue samples by several independent groups. The concentration of yeast mannan needed to inhibit killing of macrophage-engulfed *E. coli* in these studies was relatively high (1 mg/mL). Concentrations of up to 10 ng/mL have been reported in the blood in severe Candida septicaemia, but this is of little relevance to CD. The evidence that diluted culture supernatants from *M. paratuberculosis* isolates are able to inhibit killing of *E. coli* by adherent monocytes and macrophages suggests that local concentrations of released inhibitors may be sufficient in the vicinity of *M. paratuberculosis* to have a similar effect in vivo within CD granulomata where these bacteria are concentrated.

The explanation for and possible functional importance of the association between ASCA positivity and CD have been difficult to elucidate. There are problems of reproducibility between different assays, and positive results are also seen in celiac disease, possibly implying an association with increased intestinal permeability.
ASCA has also proved detectable in 20%-25% of first-degree relatives of patients with CD and has been shown to precede clinical diagnosis of CD. This might be interpreted as implying some genetic association, but, alternatively, it could imply a genetic predisposition to infection/invasion of the mucosa by an organism that expresses the mannan epitope. The association between ASCA positivity and deficiency of human mannan-binding lectin, although unconfirmed, could be one such genetic predisposition.

Bacterial pathogens have evolved several strategies to escape phagocytic killing by macrophages including avoidance of phagocytosis, inhibition of fusion of bacteria-containing phagosomes with lysosomes, escape from the phagosome, resistance to the antimicrobial environment of the mature phagolysosome, and escape from autophagic recognition. Mannan-induced inhibition of bacterial killing is an additional mechanism by which phagocytosed bacteria may survive and replicate within macrophages. It represents a plausible mechanism for acquired phagocyte dysfunction in CD as an alternative or addition to the genetic defects in autophagy that have recently been described in association with CD.

References

34. Standaert-Vitsa A, Jouault T, Vandewalle P, et al. Candida albicans is an immunogen for anti-Saccharomyces cerevisiae anti-

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MANNAN INHIBITS KILLING OF CD E COLI 1497

Alimentary Tract

Gastroenterology

Saccharomyces cerevisiae

BASIC

endotoxin

saccharide

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