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Replication of Colonic Crohn's Disease Mucosal *Escherichia coli* Isolates within Macrophages and Their Susceptibility to Antibiotics▽

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There is increasing evidence that *Escherichia coli* organisms are important in Crohn's disease (CD) pathogenesis. In CD tissue they are found within macrophages, and the adherent-invasive CD ileal *E. coli* isolate LF82 can replicate inside macrophage phagolysosomes. This study investigates replication and antibiotic susceptibility of CD colonic *E. coli* isolates inside macrophages. Replication of CD colonic *E. coli* within J774-A1 murine macrophages and human monocyte-derived macrophages (HMDM) was assessed by culture and lysis after gentamicin killing of noninternalized bacteria and verified by electron microscopy (EM). All seven CD colonic isolates tested replicated within J774-A1 macrophages by 3 h (6.36-fold ± 0.7-fold increase; $n = 7$ isolates) to a similar extent to CD ileal *E. coli* LF82 (6.8-fold ± 0.8-fold) but significantly more than control patient isolates (5.2-fold ± 0.25-fold; $n = 6$; $P = 0.006$) and *E. coli* K-12 (1.0-fold ± 0.1-fold; $P < 0.0001$). Replication of CD *E. coli* HM605 within HMDM (3.9-fold ± 0.7-fold) exceeded that for K-12 (1.4-fold ± 0.2-fold; $P = 0.03$). EM showed replicating *E. coli* within macrophage vacuoles. Killing of HM605 within J774-A1 macrophages following a 3-h incubation with antibiotics at published peak serum concentrations (C_{max}) was as follows: for ciprofloxacin, 99.5% ± 0.2%; rifampin, 85.1% ± 6.6%; tetracycline, 62.8% ± 6.1%; clarithromycin, 62.1% ± 5.6% (all $P < 0.0001$); sulfamethoxazole, 61.3% ± 7.0% ($P = 0.0007$); trimethoprim, 56.3% ± 3.4% ($P < 0.0001$); and azithromycin, 41.0% ± 10.5% ($P = 0.03$). Ampicillin was not effective against intracellular *E. coli*. Triple antibiotic combinations were assessed at 10% C_{max}, with ciprofloxacin, tetracycline, and trimethoprim causing 97% ± 0.0% killing versus 86% ± 2.0% for ciprofloxacin alone. Colonic mucosa-associated *E. coli*, particularly CD isolates, replicate within macrophages. Clinical trials are indicated to assess the efficacy of a combination antibiotic therapy targeting intramacrophage *E. coli*.

There is growing evidence that Crohn's disease (CD) results from a defective or inappropriate response to the gut microbiota. Patients with CD respond to diversion of the fecal stream, with inflammation recurring after reinstillation of luminal contents (16, 55). Enteric bacteria or their products have been found within the inflamed mucosa of patients with CD (25), and patients with CD commonly have circulating antibodies against bacterial antigens, particularly flagellin (31, 64) and outer membrane proteins (37).

It follows that antibiotics ought to represent a logical approach to therapy; however, there is relatively weak evidence for their efficacy in CD. Controlled trials have shown some benefit with ciprofloxacin (50), metronidazole (50), and rifaximin (49), and uncontrolled trials have shown promise with ciprofloxacin (6, 24), clarithromycin (29, 63), metronidazole (24), and rifabutin (63). The imidazoles, metronidazole (56) and ornidazole (57), are both effective in the prevention of postoperative recurrence of CD. However, corticosteroids are widely used as first-line treatment, and a controlled trial has shown no advantage from administration of ciprofloxacin and metronidazole in combination to patients receiving budesonide (66). However, until the target organism(s) and its site (intracellular or extracellular) are known, the choice of antibiotics can only be arbitrary. Moreover, the use of a single antibiotic in a condition where there is probably a high potential for reinfection is likely to result in antibiotic resistance.

Evidence is accumulating that *Escherichia coli* may represent an important target organism and that it may be internalized within macrophages and hence inaccessible to antibiotics that penetrate cells poorly. At least six independent studies have now reported an increased presence of mucosa-associated *E. coli* in CD tissue (13, 14, 28, 35, 62, 69). This includes a recent study, by Kotlowski and colleagues (28), where ribosomal intergenic spacer analysis was used to screen for any bacterial DNA sequences that were overexpressed in inflammatory bowel disease tissue, with the finding of selective overexpression of *E. coli* sequences in both CD and ulcerative colitis. One group has reported selective growth of the *E. coli* from distal ileal mucosal samples in CD patients with both early and chronic ileal disease (13, 14), while other investigators have reported on colonic mucosal samples (28, 35, 62, 69). Moreover, *E. coli* DNA has been found within CD granulomas cut from tissue sections using laser dissection microscopy (58). The

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† Sadly, Tony Hart passed away after the original submission. He was an inspirational colleague who is greatly missed.

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CD *E. coli* isolates lack classical pathogenicity genes, apart from adhesins, but adhere to and invade intestinal epithelial cell lines in vitro (13, 35, 62). There is little direct evidence of their presence within normal human intestinal epithelial cells in vivo, and it seems likely that, as for other bona fide pathogens such as *Salmonella* spp. and *Shigella* spp. (60), the specialized M cells in the dome epithelium overlying Peyer's patches and colonic lymphoid follicles represent their most probable portal of entry into the mucosa. In CD tissue, *E. coli* organisms have most convincingly been demonstrated within macrophages (30, 58). *E. coli* antigens have also been identified in macrophages within the lamina propria, in granulomas, and in the germinal centers of mesenteric lymph nodes in patients with CD (10, 30). A CD ileal *E. coli* isolate, LF82, has been shown to replicate within the mature phagolysosomes of macrophages in vitro (8) and to induce giant cell formation (36); but this was the most invasive of the ileal isolates previously described, and there has been no similar study of CD colonic mucosal isolates.

In this study, we have investigated the ability of CD colonic mucosal isolates to replicate within macrophages. We have then assessed a panel of antibiotics for their efficacy against *E. coli* found within macrophages in order to assist rational design of future trials of antibiotic therapy.

MATERIALS AND METHODS

Cell culture. The murine macrophage-like cell line J774-A1 was obtained from the European Collection of Animal Cell Culture (catalogue number 85011428; Public Health Laboratory Service, Wiltshire, United Kingdom). Cells were maintained at 37°C in 5% CO_2 and 95% air in RPMI 1640 medium containing 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 4 mM glutamine.

Isolation of HMDMs. Heparinized blood was obtained from consenting, healthy blood donors from the National Blood Transfusion Service. The study was approved by the Liverpool Research Ethics Committee. Human venous blood mononuclear cells were isolated from buffy coats by the Ficoll-Hypaque procedure (18). Adherent monocytes were obtained by overnight culture on 12-well plastic culture plates in RPMI medium supplemented with 10% (vol/vol) fetal calf serum, and 2% (wt/vol) L-glutamine at 37°C in an atmosphere of 95% air and 5% CO_2. Nonadherent lymphocytes were removed by washing the culture wells with serum-free RPMI medium. Human monocyte-derived macrophages (HMDMs) were obtained by further culture of adherent monocytes in complete RPMI medium containing 50 U/ml granulocyte macrophage colony stimulating factor for 5 to 7 days.

Materials. The following antibiotics were obtained from Sigma (Poole, United Kingdom): ampicillin, azithromycin, ciprofloxacin, gentamicin, rifampin, sulfamethoxazole, tetracycline, and trimethoprim. Clarithromycin (Abbott Laboratories Ltd., Queenborough, United Kingdom) suitable for intravenous administration was used. A ToxiLight bioassay cytotoxicity kit was obtained from Cambrex Bioscience (Rockland, ME). Paraformaldehyde and glutaraldehyde were obtained from Agar Scientific (Stansted, United Kingdom).

Bacterial strains and growth conditions. *E. coli* cells were previously isolated from colonic mucosal biopsies of patients with CD and a control population consisting of patients with irritable bowel syndrome or sporadic polyps (35). The *E. coli* isolates were classified according to their presence within the mucus layer (mucus associated) or beneath it (mucosa associated) after removal of the mucus layer by dithiothreitol treatment. Fourteen isolates were studied (eight CD and six control patient mucosa-associated *E. coli* isolates) plus the nonpathogenic reference strain *E. coli* K-12. The CD *E. coli* strains were isolated from six different CD patients with colonic CD (isolates HM154, HM580, HM605, and HM615), ileo-colonic CD (HM413 and HM419), and ileal CD (HM95). None of the six patients was receiving antibiotics. *E. coli* strain LF82, isolated from a chronic ileal lesion of a French patient with CD, was a kind gift from A. Darfeuille-Michaud, Université Clermont I, Pathogénie Bactérienne Intestinale, Clermont-Ferrand, France (14). HM419, HM580, HM605, and HM615 and the control mucus-associated strains HM428, HM454, and HM456 all showed mannose-resistant hemagglutination, a property that correlates strongly with adherence to colon epithelial cells (35). Of the CD isolates, HM95, HM580, and HM605 have been shown to adhere to and invade I-407 intestinal epithelial cells while HM615 adheres but does not invade, and HM154 neither invades nor adheres (35). Phylogenetic typing using the method of Clermont et al. (11) has shown that all *E. coli* isolates tested are in phylogenetic group B2 except for HM428, HM463, HM484, HM580 (in group D), and HM154 (group B1). At the time of this study, no phylogenetic grouping or adherence/invasion properties relating to I-407 cells were available for HM413 and HM419. All the CD *E. coli* strains express the type 1 adhesin gene *fimH*, with some positive for other adhesins such as *pap* and *afa* (34, 35). The control *E. coli* isolates have not been tested for the presence of pathogenicity genes. Isolates were maintained in culture on Columbia agar plates with overnight incubation in air at 37°C.

Replication of *E. coli* within J774-A1 cells and HMDMs. J774-A1 cells were seeded into 24-well tissue culture plates (Corning/Costar, High Wycombe, United Kingdom) at 10^5 cells per well and maintained in RPMI 1640 medium and used 24 h later. The monolayers were washed twice with sterile phosphate-buffered saline (PBS). Prior to infection of cells, bacteria were washed three times, resuspended in antibiotic-free RPMI 1640 medium, and added to cells at a multiplicity of infection of 10 (equivalent to 4×10^6 CFU/ml). Following a 2-h incubation at 37°C to allow internalization, cell monolayers were washed three times in PBS to remove nonadherent bacteria and treated with fresh culture medium containing 20 μg/ml gentamicin for 1 h to kill extracellular bacteria. Following this, cells were washed with sterile PBS and replaced with fresh medium containing 20 μg/ml gentamicin and incubated for a further 3 h at 37°C to allow replication of internalized bacteria. Subsequently, the monolayers were washed three times with PBS, sterility of the third wash was confirmed by culture, and cells were lysed by adding deionized water containing 1% (vol/vol) Triton X-100 for 5 min to release internalized bacteria. Studies showed that incubation of *E. coli* with this concentration of Triton X-100 for up to 60 min had no significant affect on microbial viability. Tenfold dilutions of the cell lysates were made, and 50 μl from each dilution was plated onto nutrient agar plates. Plates were incubated at 37°C, and CFU were counted after 24 h as a measure of viable bacteria. To calculate relative replication, the viable counts at the end of the 3-h incubation period were compared to the number of viable CFU obtained immediately after a 1-h gentamicin treatment. A similar protocol was used to assess replication of the CD isolate HM605 and *E. coli* K-12 in HMDMs.

Efficacy of antibiotics against internalized CD mucosa-associated *E. coli*. To assess the efficacy of antibiotics against internalized CD *E. coli*, a representative adherent and invasive strain, HM605, was used. Previous studies using the disc diffusion method for antimicrobial susceptibility testing on Iso-Sensitest agar (Oxoid, Basingstoke, United Kingdom) had shown that, of 77 Crohn's disease *E. coli* isolates tested, antibiotic sensitivities were as follows: gentamicin, 100%; ampicillin, 95%; chloramphenicol, 62%; trimethoprim, 100%; nalidixic acid, 100%; tetracycline, 67%; and penicillin, 0% (34). For this study, further antibiotic sensitivities were examined using ciprofloxacin (1 μg), sulfamethoxazole (100 μg), azithromycin (15 μg), clarithromycin (5 μg), and rifampin (5 μg) discs, according to the current antimicrobial susceptibility testing disc methods of the British Society for Antimicrobial Chemotherapy (version 6.1, February 2007; http://www.bsac.org.uk/susceptibility_testing.cfm). For 64 CD *E. coli* isolates, sensitivities were as follows: ciprofloxacin, 100%; sulfamethoxazole, 77%; azithromycin, 78%; clarithromycin, 0%; and rifampin, 0%. Subsequent dose-response experiments did, however, show that both clarithromycin and rifampin had some activity against HM605.

Cells were infected with *E. coli* HM605 as described above. Following gentamicin treatment and washes, cells were treated for a further 3 h with various concentrations of ampicillin, azithromycin, clarithromycin, ciprofloxacin, gentamicin, rifampin, sulfamethoxazole, tetracycline, and trimethoprim or with buffer as a control. To assess if extending the incubation period augmented intramacrophage killing of CD *E. coli*, the initial antibiotic incubation period of 3 h was further extended to 6 h and 24 h for ciprofloxacin (a quinolone), ampicillin (a beta-lactam), and gentamicin (an aminoglycoside). As before, sterility of the third wash following 6-h and 24-h antibiotic treatments was confirmed by culture. Based on the efficacy of some antibiotics as single agents, we also assessed ciprofloxacin in combination with tetracycline and rifampin or with tetracycline and trimethoprim at peak serum concentrations (C_{max}) as well as at 10% of C_{max}. In addition, we further assessed the efficacy of antibiotics against two other CD colonic *E. coli* strains (HM580 and HM615) and against the well-characterized CD ileal isolate, LF82. Cells were lysed and plated as described above, CFU were quantified, and the percentages of bacteria killed by the antibiotics were determined in comparison with bacterial counts obtained after a 3-h incubation in the absence of antibiotics.

Transmission electron microscopy (EM) of macrophages. Subsequent to infection, as described above, J774-A1 cells were fixed in 2% glutaraldehyde and 4% paraformaldehyde in PBS. Cells were then washed in PBS (5 min), twice in PBS–0.15

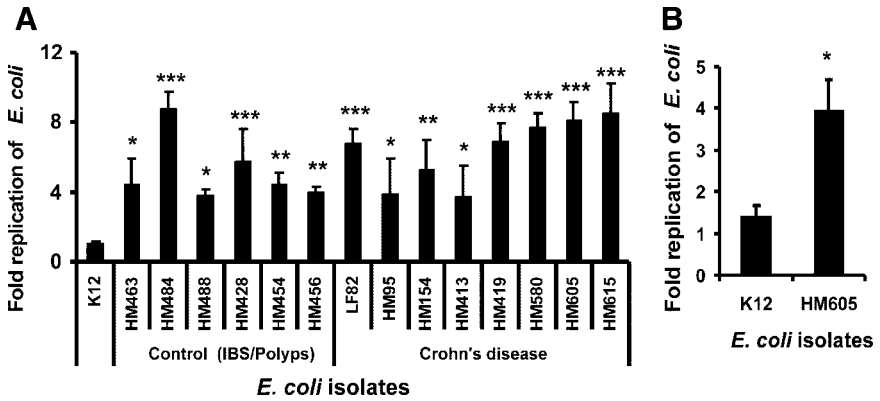

FIG. 1. CD mucosa-associated *E. coli* isolates replicate effectively inside J774-A1 macrophages and HMDMs. (A) Replication of *E. coli* strains isolated from CD ($n = 8$) and control patients ($n = 6$) within J774-A1 murine macrophages (mean ± standard error of the mean) compared to the nonpathogenic reference strain *E. coli* K-12. Replication is shown as the relative change after 3 h of growth within macrophages. Significant differences from *E. coli* K-12 replication rates are as follows: *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ (ANOVA). (B) Increased replication of CD colonic mucosa-associated *E. coli* strain HM605 is also shown within HMDMs (mean ± standard error of the mean) compared to the reference strain *E. coli* K-12. Differences from *E. coli* K-12 replication rates were significant (*, $P < 0.05$; Mann-Whitney U test). IBS, irritable bowel syndrome.

M glycine (two times for 5 min each time), and then 1 ml of PBS was added per plate. Cells were carefully scraped into microcentrifuge tubes containing PBS and centrifuged at 9,000 × *g* for 2 min; 2% agarose was added, and cells were centrifuged at 9,000 × *g* for 2 min and then placed on ice until the agarose set. Samples were trimmed to 1-mm^3 pieces and incubated in 1% osmium tetroxide for 1 h at room temperature, followed by sequential dehydration in ethanol and acetone; samples were then mounted in araldite resin. Seventy-nanometer-thick sections were loaded onto copper grids, stained for 5 min each in Reynold's lead citrate stain and 5% uranyl acetate, washed for 1 min in distilled water, air-dried for 24 h, and examined using a FEI 120 kV Tecnai G 2 Spirit BioTWIN transmission electron microscope (FEI Company; Hillsboro, OR).

Cytotoxicity assay. Viability of macrophages on exposure to the maximum concentrations of antibiotics tested and incubation with HM605 was assessed by measuring the release of adenylate kinase into the supernatant over 3 h using a ToxiLight bioassay kit as per the manufacturer's instructions.

Quantification of cellular protein. Protein concentrations of macrophage lysates following exposure to the maximum concentrations of antibiotics tested were determined using a bicinchoninic acid assay (Sigma) as per the manufacturer's instructions.

Determination of MICs. For each *E. coli* isolate used in the macrophage replication studies, the MIC was determined using the Etest antibiotic concentration gradient method. Nutrient agar plates (Oxoid Ltd., Basingstoke, United Kingdom), 90 mm in diameter, were inoculated by swabbing with an initial *E. coli* suspension (0.5 McFarland standard; in sterile saline). AB Biodisk Etest strips (Biostat; Stockport, United Kingdom) for each antibiotic were applied to the inoculated agar as per the manufacturer's instructions and incubated at 35°C with 5% CO_2 for 24 h. The MIC was defined as the value where the growth inhibition ellipse intersected the strip. *E. coli* ATCC 25922, obtained from the American Type Culture Collection (Manassas VA), was used as a quality control isolate for Etest strips, where appropriate.

Determination of MBCs. CD *E. coli* HM605 suspended in RPMI medium was used for determination of the minimum bactericidal concentration (MBC), which was defined as the lowest concentration of each drug causing >99.9% reduction in growth.

Statistical analysis. Independent sample groups were assessed for normality and equality of variances. For multiple treatment groups, one-way analysis of variance (ANOVA) was employed; in the case of significant differences, pairwise comparisons of treatment means (StatsDirect version 2.6.2; Sale, United Kingdom) were then used. Differences were considered significant at *P* values of <0.05.

RESULTS

CD *E. coli* isolates replicate inside J774-A1 macrophages. By 3 h, all eight colonic mucosal CD *E. coli* isolates tested were better able to replicate inside J774-A1 macrophages than the laboratory strain *E. coli* K-12 ($P < 0.0001$, ANOVA) (Fig. 1A). The CD ileal isolate LF82 replicated at a level (6.8-fold ± 0.8-fold) similar to that of the other seven CD colonic isolates (6.36-fold ± 0.7-fold) in comparison to *E. coli* K-12 (1.0-fold ± 0.1-fold). Six mucosal *E. coli* strains isolated from control patients showed greater replication (5.2-fold ± 0.25-fold) within macrophages than *E. coli* K-12 ($P < 0.0001$) (Fig. 1A). These six control isolates showed significantly less replication than the eight CD isolates tested ($P = 0.006$, Kruskal-Wallis ANOVA).

CD *E. coli* isolates also replicate within HMDMs. The CD *E. coli* HM605 isolate was also better able to replicate inside HMDM than the laboratory strain *E. coli* K-12. By 3 h, HM605 replicated by 3.9-fold ± 0.7-fold compared with 1.4-fold ± 0.2-fold for *E. coli* K-12 ($P = 0.03$, Mann Whitney U test) (Fig. 1B).

CD *E. coli* isolates replicate within macrophage vacuoles. Three hours after infection, HM605 was observed inside vacuoles within J774-A1 macrophages (Fig. 2). Infected cells contained large vacuoles with the appearance of phagolysosomes, some of which had fused. Bacteria were detected that appeared to be undergoing replication. No internalized bacteria were observed free in the cytoplasm. The presence of bacteria within vacuoles did not affect the nuclear or cytoplasmic membrane morphology, and the macrophages retained an otherwise normal appearance.

Susceptibility of CD *E. coli* to antibiotics in the absence of macrophages. The MBCs for CD *E. coli* HM605 and the commonly observed C_{max} values seen in patients receiving conventional doses of the antibiotics are listed in Table 1.

An Etest for MIC determination showed that the eight CD *E. coli* and six control patient *E. coli* isolates were sensitive to ciprofloxacin (MIC of ≤1 μg/ml; range, 0.004 to 0.19 μg/ml). Similarly, all showed either sensitivity (MIC of ≤ 2μg/ml) or an intermediate effect (MIC of 3 μg/ml) for azithromycin. Six of eight CD *E. coli* and five of six control patient isolates were sensitive for trimethoprim and tetracycline (both, MIC of ≤4). For sulfamethoxazole, five of eight CD and two of six control

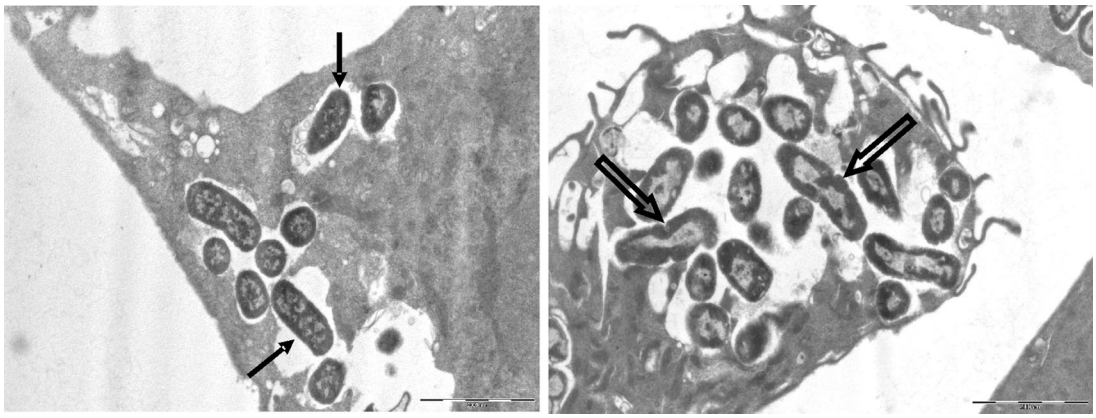

FIG. 2. CD *E. coli* strain HM605 replicates in vacuoles of J774-A1 macrophages. Images are EM of J774-A1 macrophages infected with CD *E. coli* strain HM605 as described in Materials and Methods. Extracellular bacteria were eliminated with gentamicin. Filled arrows indicate HM605 within vacuoles. HM605 survives and replicates within vacuolar structures without affecting cellular morphology. Hollow arrows indicate replicating HM605. Bar, 2 μm.

isolates showed sensitivity (MICs of ≤256). Two of eight CD *E. coli* isolates and three of six control patient isolates were resistant for ampicillin (MICs of >16); all others were sensitive (MIC of <8). All eight CD and six control isolates tested had a MIC of ≥16 μg/ml to clarithromycin. One sole isolate (CD *E. coli* HM413) showed an intermediate response (MIC of 2) to rifampin; for all other isolates tested, the MIC was ≥4.

For laboratory strain *E. coli* K-12, sensitivity was seen with all antibiotics tested excepting rifampin (intermediate; MIC of 3 μg/ml) and clarithromycin (MIC of 24 μg/ml). *E. coli* ATCC 25922 was sensitive to ampicillin, ciprofloxacin, sulfamethoxazole, trimethoprim, and tetracycline; MICs were all within Etest quality control ranges.

Azithromycin, ciprofloxacin, clarithromycin, rifampin, sulfamethoxazole, tetracycline, and trimethoprim but not ampicillin are effective against HM605 CD *E. coli* within J774-A1 macrophages. Azithromycin, ciprofloxacin, clarithromycin, rifampin, tetracycline, and trimethoprim were all effective against CD *E. coli* HM605 within J774-A1 macrophages (Fig. 3). Complete killing of intramacrophage HM605 was achieved only with ciprofloxacin. EM confirmed the presence of bacterial debris within vacuoles in the presence of ciprofloxacin (Fig. 4). At C_{max}, ciprofloxacin induced maximal intramacrophage killing of HM605, followed by rifampin, sulfamethoxazole, tetracycline, trimethoprim, and the macrolides (Table 1). Ciprofloxacin-induced killing was already maximal (99.5%) by 3 h. Ampicillin, after a 3-h treatment, was effective only against extracellular but not against internalized HM605 (Fig. 3). Extending this treatment period to 6 and 24 h did not enhance intracellular killing of HM605 by ampicillin. Although the ampicillin MIC was >256 μg/ml for HM605 (Table 1), lower concentrations were seen to inhibit growth in culture medium as assessed by colony counts (Fig. 3). Gentamicin was also tested for its effect on internalized *E. coli* since there is some evidence that small amounts of gentamicin may penetrate macrophages (17). No significant killing was seen at C_{max} (22) (18 μg/ml) following a 3-h (7.8% ± 15.2% killing; $n = 6$) or 6-h (0.3% ± 11.4%; $n = 3$) incubation with gentamicin. However, following a 24-h incubation, there was a significant reduction in intracellular bacterial viability (54.8% ± 3.8%; $n = 3$; $P < 0.0001$), indicating some penetration of gentamicin with prolonged exposure. At 10% of C_{max} the combination of ciprofloxacin with either tetracycline and trimethoprim or tetracycline and rifampin (97.0% ± 0.0% and 94.0% ± 1.0%,

TABLE 1. Efficacy of antibiotics against internalized CD *E. coli* HM605 in J774-A1 macrophages at C_{max}

Antibiotic (n)[a]	Dose (mg [route])[b]	C_{max} (μg/ml)[c]	Reference	MBC (μg/ml)[d]	MIC (μg/ml)	% Intracellular HM605 killing at C_{max} (mean ± SEM)[e]
Ampicillin (9)	500 (p.o.)	3.2	23	>100	>256	11.1 ± 5.4 (12.3 ± 7.8)
Azithromycin (3)	500 (p.o.)	0.4	9	5	3	*41.0 ± 10.5* (NA)
Ciprofloxacin (9)	400 (i.v.)	4	61	0.3	0.006	**99.5 ± 0.2** (99.5 ± 0.3)
Clarithromycin (6)	500 (p.o.)	3	32	3	16	**62.1 ± 5.6** (73.4 ± 3.2)
Rifampin (6)	600 (p.o.)	10.5	48	3	6	**85.1 ± 6.6** (94.4 ± 1.0)
Sulfamethoxazole (6)	800 (p.o.)	40–60	51	NA	>1024	**61.3 ± 7.0** (65.3 ± 5.4)
Tetracycline (6)	250 (p.o.)	1.7	44	1	2	**62.8 ± 6.1** (55.9 ± 4.4)
Trimethoprim (6)	160 (p.o.)	2.3	67	100	>32	**56.3 ± 3.4** (64.3 ± 8.4)

[a] n, number of experiments.
[b] p.o., orally; i.v., intravenously.
[c] Concentrations corresponding to the peak serum levels of the respective antibiotics following administration of conventional doses in humans based on published literature. The efficacy of various antibiotics on killing of internalized CD *E. coli* HM605 within J774-A1 macrophages at C_{max} values is listed.
[d] Ampicillin caused 99.8% killing at the highest concentration tested (100 μg/ml). NA, no available data.
[e] Significant differences from antibiotic-free survival rates are indicated as follows: italics, $P < 0.05$; boldface, $P < 0.001$ (ANOVA). Values in parentheses represent data corrected for macrophage numbers at the end of incubation, as estimated by total cell protein assay (all, $n = 3$). NA, no available data.

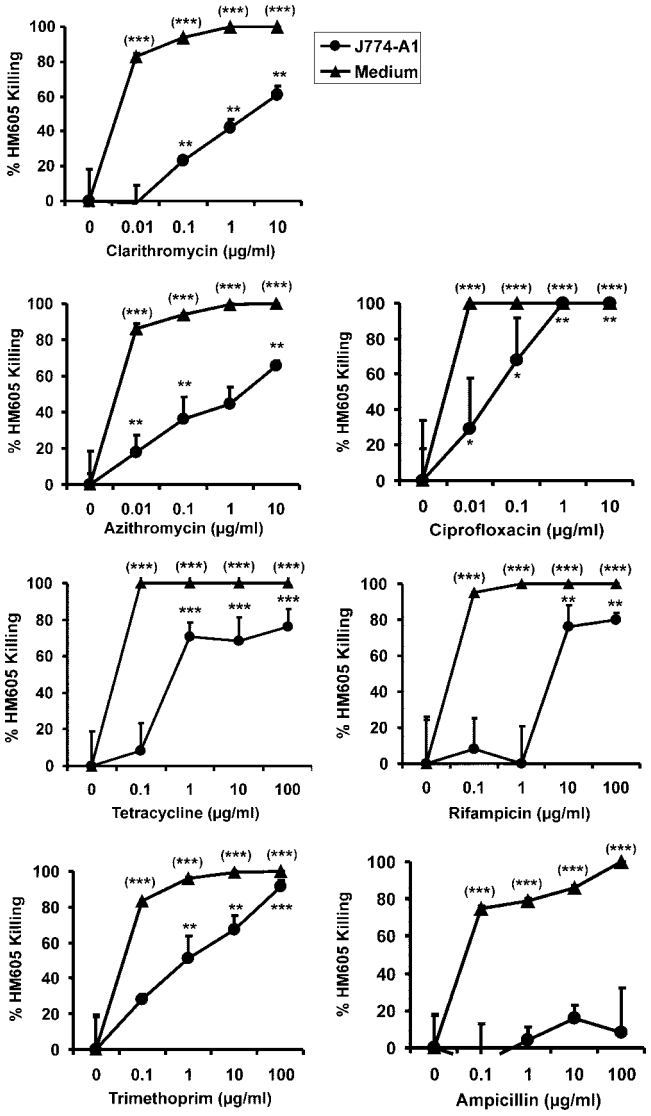

FIG. 3. Azithromycin, ciprofloxacin, clarithromycin, rifampin, tetracycline, and trimethoprim kill CD *E. coli* HM605 within macrophages. Values are the percentages (mean ± standard error of the mean, $n = 3$) of CD *E. coli* strain HM605 bacteria killed by the indicated antibiotics. Similar concentrations of antibiotics also effectively killed HM605 in medium alone. Ampicillin did not affect viability of intracellular HM605 within macrophages. However, similar concentrations of ampicillin did cause killing of HM605 in medium alone. Significant differences from survival of HM605 within J774-A1 macrophages are indicated as follows: *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$. (***), $P < 0.001$ for survival of HM605 in medium alone.

respectively) achieved higher intramacrophage killing of *E. coli* than ciprofloxacin alone (86.0% ± 2.0%; $P < 0.01$) (Fig. 5). Additional experiments to assess the efficacy of antibiotics (at 10% of C_{max}) against the replication of four of the CD *E. coli* isolates used in this study (HM580, HM605, HM615, and LF82) showed that all strains were similarly susceptible to the antibiotics tested within J774-A1 macrophages (Table 2).

Neither the presence of internalized *E. coli* HM605 nor the highest concentrations of the antibiotics tested had any toxic effect on the macrophages as assessed by release of adenylate kinase, which was always 90 to 110% of the untreated control value. Similarly, macrophage numbers at the end of incubation, as estimated by total cell protein assay, were consistent across controls (0.076 ± 0.011 mg of protein/ml [mean ± standard deviation]) and antibiotic-treated samples (0.085 ± 0.014 mg/ml).

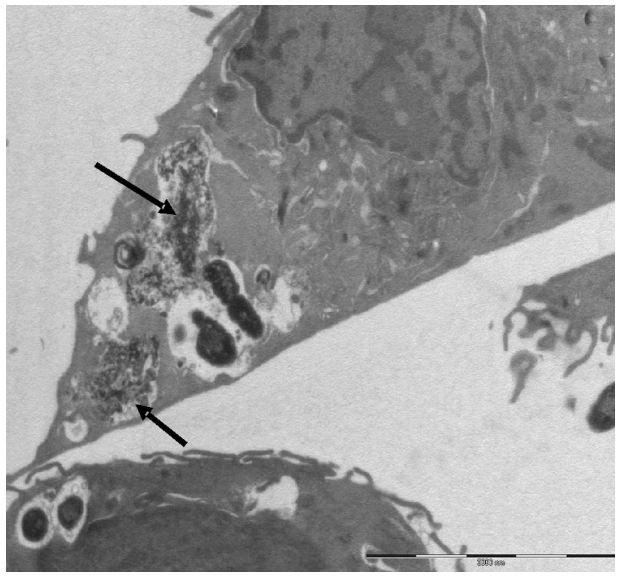

FIG. 4. Ciprofloxacin affects viability of CD *E. coli* strain HM605 in J774-A1 macrophages: EM of J774-A1 macrophages infected with CD *E. coli* HM605 in the presence of 0.1 μg/ml ciprofloxacin. Extracellular bacteria were eliminated with gentamicin. Arrows indicate the presence of bacterial debris within vacuoles. Bar, 5 μm.

DISCUSSION

CD colonic mucosa-associated *E. coli* isolates are shown to replicate inside vacuoles within macrophages without any ap-

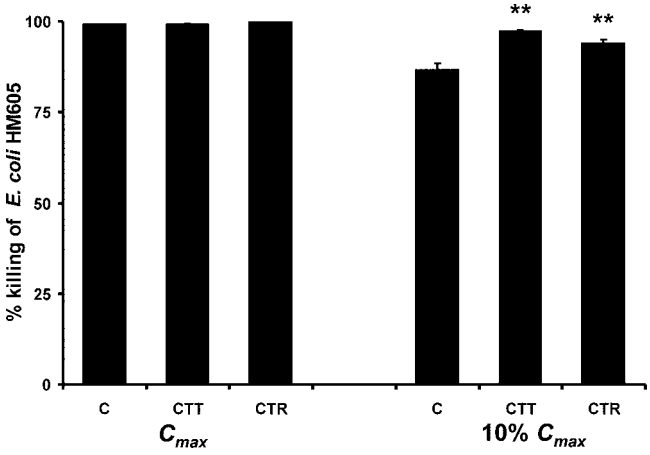

FIG. 5. The combination of either ciprofloxacin, trimethoprim, and tetracycline (CTT) or ciprofloxacin, trimethoprim, and rifampin (CTR) was able to achieve better killing than ciprofloxacin alone at 10% of C_{max}. **, $P < 0.01$ in comparison to killing with ciprofloxacin alone. There was no significant difference in killing of HM605 within macrophages between single and combination antibiotics at C_{max}.

TABLE 2. Efficacy of antibiotics against internalized CD *E. coli* strains in J774-A1 macrophages at 10% C_{max}

Antibiotic	% Intracellular killing of the indicated *E. coli* strain at 10% C_{max} (mean ± SEM)[a]			
	LF82	HM580	HM605	HM615
Ampicillin	10.9 ± 7.8	9.6 ± 11.6	20.4 ± 8.4	13.8 ± 15.4
Ciprofloxacin	87.6 ± 1.9***	90.4 ± 1.0***	91.3 ± 4.8***	83.8 ± 2.4***
Clarithromycin	55.2 ± 6.0***	57.5 ± 8.7***	33.5 ± 2.6**	81.8 ± 2.1***
Rifampin	32.8 ± 7.4 **	35.8 ± 9.3 **	32.6 ± 9.5 **	80.2 ± 2.1***
Sulfamethoxazole	63.2 ± 6.1**	58.7 ± 7.1**	39.8 ± 17.0**	71.5 ± 17.2***
Tetracycline	64.2 ± 6.2***	62.4 ± 4.0***	44.8 ± 8.3***	69.6 ± 8.2***
Trimethoprim	50.2 ± 6.7***	46.9 ± 6.1***	49.1 ± 4.9***	61.7 ± 3.1***

[a] Significant differences from antibiotic-free survival rates are indicated as follows: *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ ($n = 3$). Published C_{max} values are listed in Table 1.

parent toxic effect on the macrophages and in this respect resemble the previously characterized CD ileal isolate LF82. The antibiotics azithromycin, ciprofloxacin, clarithromycin, rifampin, sulfamethoxazole, tetracycline, and trimethoprim are effective against these macrophage-internalized *E. coli*. The combination of ciprofloxacin, tetracycline, and trimethoprim is particularly effective.

Neutrophils normally serve as the first line of defense against microorganisms that have invaded from the intestinal lumen (3). In CD, there is evidence of neutrophil dysfunction, as shown by abnormally low neutrophil accumulation and lower interleukin-8 production following trauma to rectum, ileum, or skin (33). The rare inherited conditions chronic granulomatous disease (27) and glycogen disease type 1b (12), both associated with well-characterized defects in neutrophil killing of bacteria, are also associated with Crohn's-like intestinal disease. Defective neutrophil clearance of intramucosal bacteria is likely to lead to uptake of the bacteria by intestinal macrophages, and this is supported by the evidence for *E. coli* within CD tissue macrophages as shown by immunohistochemistry (30) and by PCR demonstration of ribosomal DNA in laser-dissected granulomas (58). This will usually trigger an inflammatory cascade that could result in either elimination of the organism or facilitation of further bacterial invasion (1), and this might explain some of the unpredictable variation in the natural history of CD. If the bacteria are not killed by the macrophages and do not induce apoptosis of the macrophages themselves, it is highly plausible that a chronic granulomatous reaction, typical of that seen in CD, would result. Intestinal macrophages are distinct in phenotype and function from blood monocytes in that they retain avid scavenger and host defense functions but acquire profound inflammatory anergy (65). It would be interesting to study the interaction of CD *E. coli* with intestinal macrophages. J774-A1 murine macrophages have been used in previous studies that reported LF82 replication in macrophages (8).

The CD *E. coli* isolates are shown to be able to survive and replicate within macrophage vesicles. This has also been shown for the French CD ileal isolate LF82 (8). However, other *E. coli* colonic isolates present in the colonic mucus of healthy individuals, not just those with CD, are also found here to replicate better within macrophages than the laboratory control *E. coli* K-12, and so this may in part reflect the unaggressive phenotype of K-12; moreover, there was considerable overlap between the replication within macrophages of the CD and control patient isolates. Macrophages are generally poorer at bacterial killing than neutrophils—the "professional" killers (3)—so it may be the poor clearance of *E. coli* from the CD mucosa first by antimicrobial peptides, such as defensins (42, 71), and then by neutrophils (3) and their consequent uptake by macrophages rather than the pathogenic features of the bacteria themselves that determine their chronic replication within tissue macrophages. This may be particularly important in patients who have inherited polymorphisms in the autophagy genes that have recently been shown to associate with increased risk for CD (47, 52).

Bacterial pathogens have evolved several strategies to escape phagocytic killing by macrophages including avoidance of phagocytosis (19, 20), inhibition of fusion of bacteria-containing phagosomes with lysosomes (4), escape from the phagosome (26), resistance to the antimicrobial environment of the mature phagolysosome (7), or avoidance of autophagic recognition (45). Some bacteria can affect rates of phagocyte apoptosis to enhance their survival (15). The CD ileal adherent-invasive *E. coli* isolate LF82 was shown to undergo normal interaction with host endomembrane organelles and to replicate within the acidic environment of vacuolar phagolysosomes (8), with the resulting giant-cell formation (36). The colonic CD adhesive *E. coli* isolates described here also replicate readily within macrophage vacuoles. Further work is needed to characterize the mechanisms by which the CD-associated *E. coli* avoid killing by macrophages while failing to induce macrophage apoptosis.

Successful eradication of bacteria by antibiotics is dependent on a number of pathogen-, drug-, and patient-related factors. C_{max} values, area under the plasma concentration-versus-time curve, and the duration for which the drug plasma concentration exceeds its MIC all serve as useful indicators of antibiotic efficacy (59). Clinical outcome also depends on microbial killing and the persistence of antibacterial effects after plasma concentrations have fallen below the MIC for the target pathogen (postantibiotic effect) (41). The pharmacokinetic property that best predicts efficacy, however, varies for each drug. Thus, it is difficult to predict in vivo efficacy of antibiotics using a single parameter. For the antibiotics tested here, we have assessed efficacy against CD *E. coli* isolates at C_{max} as well as at 10% of $C_{max.}$ By these criteria ciprofloxacin, clarithromycin, tetracycline, and trimethoprim exhibit significant antibacterial effects at much lower concentrations than $C_{max.}$ Apart from ciprofloxacin, none of the other antibiotics studied achieved complete killing at conventionally seen C_{max} levels.

High intracellular concentration within macrophages may be particularly relevant for antibiotic efficacy in CD. Azithromycin, fluoroquinolones, and, to a lesser extent, clarithromycin penetrate polymorphonuclear leukocytes, monocytes, lymphocytes, and macrophages and achieve high intracellular concentrations (38, 54). The intracellular antibacterial activities observed in our study are lower than would be expected on the basis of the activity of the antibiotics in medium alone and their known levels of cellular accumulation. This is in keeping with other studies that have found no direct correlation between accumulation and activity for fluoroquinolones (21, 46) or macrolides (21, 39). The reduced activity of antibiotics in the intracellular milieu is probably multifactorial and could be due to cellular compartmentalization of the drug, the acidic

phagolysosomal environment, or antibiotic binding to cellular components (40).

The present in vitro study does not take into account biologically active metabolites. For instance, clarithromycin is dependent on its active metabolite 14-hydroxyclarithromycin for optimal killing of gram-negative bacteria (5), and so the in vitro assay may underestimate its effect in vivo. Moreover, the concentration of drug metabolites can vary between individuals and may be affected by liver function or by concomitantly administered drugs.

We should not ignore the possible role of anaerobic bacteria in CD pathogenesis, at least as an invading pathogen after a breach in the mucosa has occurred. CD patients commonly have circulating antibodies against clostridial flagellin (31) and have a greater proportion of *Bacteroides fragilis* in their mucosal biofilm (70). Metronidazole has been shown to reduce postoperative recurrence of CD (56) and to have some efficacy against colonic CD (68). The interaction between anaerobic bacteria and macrophages has been relatively little studied, but *Clostridium perfringens* has been shown to be toxic to macrophages (43), and *B. fragilis* has been shown to have an intriguing synergistic effect in mixed infections with *E. coli* that is probably due to suppression of macrophage phagocytosis by *B. fragilis* (53). It may therefore be appropriate to include anaerobes such as *B. fragilis* as targets for antibiotic therapy in CD.

Clinical trials in CD using combinations of antibiotics are now indicated from our data. The possibility has to be acknowledged that there are potential deleterious effects including overgrowth of *Clostridium difficile* and the inadvertent reduction of some possibly beneficial bacteria. The combination should probably include ciprofloxacin because of its superior in vitro efficacy plus at least one of the other antibiotics—clarithromycin, rifampin, tetracycline, or trimethoprim—that kill *E. coli* within macrophages. Even though there are theoretical objections to using a bacteriostatic antibiotic (tetracycline) in combination with bactericidal antibiotics, doxycycline-rifampin combinations have been used with good effect in treating brucellosis (2). Based on the in vitro killing of macrophage-engulfed CD *E. coli*, some combination of ciprofloxacin, tetracycline, and/or trimethoprim would be a suitable regimen to subject to formal clinical trial.

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