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M-Cell Targeting of Whole Killed Bacteria Induces Protective Immunity against Gastrointestinal Pathogens[▽]

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As the majority of human pathogens infect via a mucosal surface, delivery of killed vaccines by mucosal routes could potentially improve protection against many such organisms. Our ability to develop effective killed mucosal vaccines is inhibited by a lack of adjuvants that are safe and effective in humans. The *Ulex europaeus* agglutinin I (UEA-I) lectin specifically binds M cells lining the murine gastrointestinal tract. We explored the potential for M-cell-targeted vaccination of whole, killed *Helicobacter pylori*, the main causative agent of peptic ulcer disease and gastric cancer, and *Campylobacter jejuni*, the most common cause of diarrhea. Oral delivery of UEA-I-agglutinated *H. pylori* or *C. jejuni* induced a significant increase in both serum and intestinal antibody levels. This elevated response (i) required the use of whole bacteria, as it did not occur with lysate; (ii) was not mediated by formation of particulate clumps, as agglutination with a lectin with a different glycan specificity had no effect; and (iii) was not due to lectin-mediated, nonspecific immunostimulatory activity, as UEA-I codelivery with nonagglutinated bacteria did not enhance the response. Vaccination with UEA-I-agglutinated, killed whole *H. pylori* induced a protective response against subsequent live challenge that was as effective as that induced by cholera toxin adjuvant. Moreover, vaccination against *C. jejuni* by this approach resulted in complete protection against challenge in almost all animals. We believe that this is the first demonstration that targeting of whole killed bacteria to mucosal M cells can induce protective immunity without the addition of an immunostimulatory adjuvant.

The majority of human pathogens infect via a mucosal surface. For example, *Helicobacter pylori* chronically colonizes the gastric mucosa, driving an inflammatory response that in some individuals results in peptic ulcer disease (17) or gastric cancer (9), while intestinal infection by *Campylobacter jejuni* is the most common cause of bacterially mediated diarrhea (38). Despite this, killed and subunit vaccines are typically delivered systemically. The main reason for this is the lack of a suitable mucosal adjuvant that is both effective and safe for use in humans. Our emerging understanding of the mucosal immune system suggests that improved protection may be achievable if vaccines could be delivered via the appropriate mucosal surface rather than by injection. Therefore, much attention is being paid to the development of novel mucosal adjuvants, as well as to strategies that can circumvent the requirement for such adjuvants.

The Peyer's patch is the main site of immune induction in the gastrointestinal tract, whereby immune complexes and antigens in the gastrointestinal lumen are sampled and delivered to underlying mucosal immune cells. To fulfill this key role, Peyer's patches have some significant adaptations. First, they possess a domed structure with no surface villi and a reduced mucus layer, to facilitate contact with antigens present in the intestinal tract (8). Second, they possess highly modified epithelial cells (M cells), which are very efficient at sampling antigen for transfer across the epithelial surface to underlying immune cells (8). These specialized M cells have reduced numbers of and shortened microvilli and are commonly in direct contact with lymphocytes and macrophages that are enfolded within pockets of their cell wall (15) in order to facilitate rapid transfer of antigen to these immune cells.

Another notable feature is that the M cells are the only cell population within the murine gastrointestinal tract that express Fucα1,2-terminal saccharides, as demonstrated by the unique binding of the lectin *Ulex europaeus* agglutinin I (UEA-I) to mouse M cells (6). Not only does UEA-I lectin bind to the apical surface of M cells, but it is rapidly endocytosed and transcytosed into Peyer's patches (7). In addition to those M cells present at the luminal surface of Peyer's patches, UEA-I also binds to murine villous M-like cells (35), which have been proposed as alternative entry points for the uptake of gut bacteria (20). As antigen uptake by M cells is a key process in the induction and regulation of the immune response to mucosal pathogens, numerous studies have used this unique glycosylation for targeted delivery to the Peyer's patches. UEA-I has previously been used to target microspheres (14) and liposomes (5) to mouse M cells, with such an approach used to induce immune responses against viruses, including human immunodeficiency virus peptides (23) and hepatitis B virus (18).

Here, we examine the effects on the mucosal and systemic immune responses, as well as the induction of protective immunity, of targeted delivery of two different pathogenic bacteria (*H. pylori* and *C. jejuni*) to gastrointestinal M cells, without the addition of an immunostimulatory adjuvant. While it is known that lectin-mediated targeting can facilitate particulate uptake by Peyer's patches (21), to our knowledge this is the first study that has explored the potential application of M-cell targeting of whole killed bacteria as a vaccine approach.

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MATERIALS AND METHODS

Bacterial culture and preparations. *H. pylori* (strains SS1 and NCTC 11637) and *C. jejuni* (strain 81-176) were initially grown on blood agar plates (Blood Agar Base no. 2, and 0.02% [vol/vol] Amphostat (Thermoelectron, Waltham, MA) and 5% horse blood (Biolabs, Australia)) in an anaerobic jar with a microaerophilic gas-generating kit (Oxoid, Basingstoke, United Kingdom) for 2 days at 37°C. *H. pylori* was then cultured in brain heart infusion (BHI) broth (Oxoid) containing 5% horse serum (JRH Biosciences), 0.02% Amphostat, and Skirrow's selective supplements under microaerophilic conditions at 37°C for 24 h. These preparations were used for lectin binding studies and for infection of mice.

For formalin fixation (29), bacteria were suspended in 0.01 M formaldehyde in phosphate-buffered saline (PBS) and adjusted to an optical density at 600 nm of 1.5. After 2 h of gentle shaking at 37°C, the bacteria were shaken overnight at room temperature, washed three times in PBS, and resuspended at 10^8 bacteria/ml.

For preparation of lysate, *H. pylori* was sonicated 30 times on ice (30-s pulses with 1-min intervals; Cell Disruptor B30 [Branson Sonic Power Co., Danbury, CT]). To confirm the absence of viable bacteria, cell lysates were plated onto blood agar plates and incubated for 5 days as described above.

Lectin-mediated bacterial agglutination. For determination of the minimum lectin concentrations required to agglutinate the bacteria, the lectins UEA-I and *Bandeiraea simplificolia* I (BS-I) (Sigma, St. Louis, MO) were dissolved in PBS at various concentrations (1,000, 200, 100, 50, 25, 10, 5, and 2 μg/ml) and 10 μl mixed with an equal volume of bacteria resuspended in PBS at 10^8 bacteria/ml. The final lectin concentrations when mixed with bacteria were 500, 100, 50, 25, 12.5, 5, 2.5, and 1 μg/ml. PBS alone was used as a control for autoagglutination of the bacteria. The bacterial suspensions were incubated for 5 s at room temperature, and then 10 μl of the mixture was transferred to a slide and examined immediately under light microscopy for bacterial agglutination.

Oral delivery of lectin-agglutinated bacteria to mice. Specific-pathogen-free female C57BL/6 and BALB/c mice (Walter and Eliza Hall Institute, Melbourne, Australia) were housed in the Veterinary Science animal house, University of Melbourne, with free access to sterilized food and water. All experiments involved age-matched 6- to 8-week-old mice and were performed under institutional Animal Ethics Committee approval.

Mice were fasted overnight prior to bacterial delivery. Lectin-agglutinated or unagglutinated bacteria (10^7 *H. pylori* or *C. jejuni* organisms) were delivered orogastrically, resuspended in 100 μl PBS. For orogastric delivery, mice were carefully restrained and a 4-cm flexible catheter (polyethylene tube of 0.96 mm outer diameter and 0.58 mm inner diameter; Tyco Electronics, PA) attached to a syringe via a 23-gauge blunt needle inserted via the oral route into the stomach. For lectin-agglutinated *H. pylori*, 10^7 bacteria were preincubated with either 20 μg of UEA-I or 50 μg of BS-I for 10 min prior to oral delivery. These lectin concentrations were used in order to produce bacterial aggregates of similar size (Fig. 1). For *C. jejuni*, 10^7 bacteria were similarly agglutinated with 100 μg UEA-I. Where present, agglutination was clearly visible by macroscopic examination but was also confirmed by light microscopy.

Quantification of antibody responses. For sera, blood was collected by cardiac puncture and left to coagulate prior to collection of sera. For intestines, a 10-cm length of the lower small intestine was opened longitudinally and the contents carefully removed. The intestinal mucosal surface was then scraped with a scalpel blade, and the collected mucus layer was weighed and then mixed with an equal volume of PBS containing complete mini-EDTA-free proteinase inhibitor cocktail (Roche Diagnostics, Mannheim, Germany).

The levels of specific antibodies were quantified by standard direct enzyme-linked immunosorbent assay (ELISA). Maxisorp immunoplates (Nunc, Roskilde, Denmark) were coated overnight with 5 μg/well of bacterial lysate in 50 μl of bicarbonate buffer (pH 9.6). After being washed with 0.05% (vol/vol) Tween 20 in PBS, wells were blocked with 1% (wt/vol) bovine serum albumin in PBS (PBS-BSA) for 30 min at room temperature. Samples were serially diluted 1/10 in PBS-BSA and 50 μl added to duplicate wells before incubation at room temperature for 1 h. After further washing, 50 μl of either horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) (Pierce, Rockford, IL) diluted 1/10,000 or horseradish peroxidase-conjugated goat anti-mouse IgA (Southern Biotech, Birmingham, AL) diluted 1/5,000 in PBS-BSA was added to each well and the plates incubated at room temperature for 1 h. Color was developed by addition of tetramethylbenzidine (Zymed, CA) and the reaction stopped after 15 min by addition of 50 μl of 1 M H_2SO_4. Absorbance was read at 450 nm and end point titers calculated.

Quantification of bacterial burdens by colony-forming assay. *H. pylori* infection levels within mouse gastric tissues were quantified by colony-forming assay. Briefly, stomachs were opened along the inner curvature and divided into two halves. One half was placed in BHI broth and homogenized (GmbH Polytron homogenizer; Kinematica, Switzerland). Tenfold serial dilutions were prepared in BHI broth and aliquots spread over GSSA selective agar plates (Blood Agar Base no. 2 with 5% horse blood, vancomycin [10 mg/ml], polymyxin B [0.33 mg/ml], bacitracin [20 mg/ml], nalidixic acid [1.07 mg/ml], and amphotericin B [5 mg/ml]). After 5 days of culture as described above, colonies were counted and the number of CFU per stomach calculated (31).

For quantifying *C. jejuni* infection levels, a 10-cm length of the lower small intestine was removed, weighed, placed in BHI broth, and then homogenized. Tenfold serial dilutions of the resulting homogenate were prepared in BHI broth, and aliquots were spread over campylobacter selective agar plates (Blood Agar Base no. 2, Skirrow's selective supplements [Oxoid], and 0.02% [vol/vol] Amphostat and 5% horse blood) and then incubated under microaerophilic

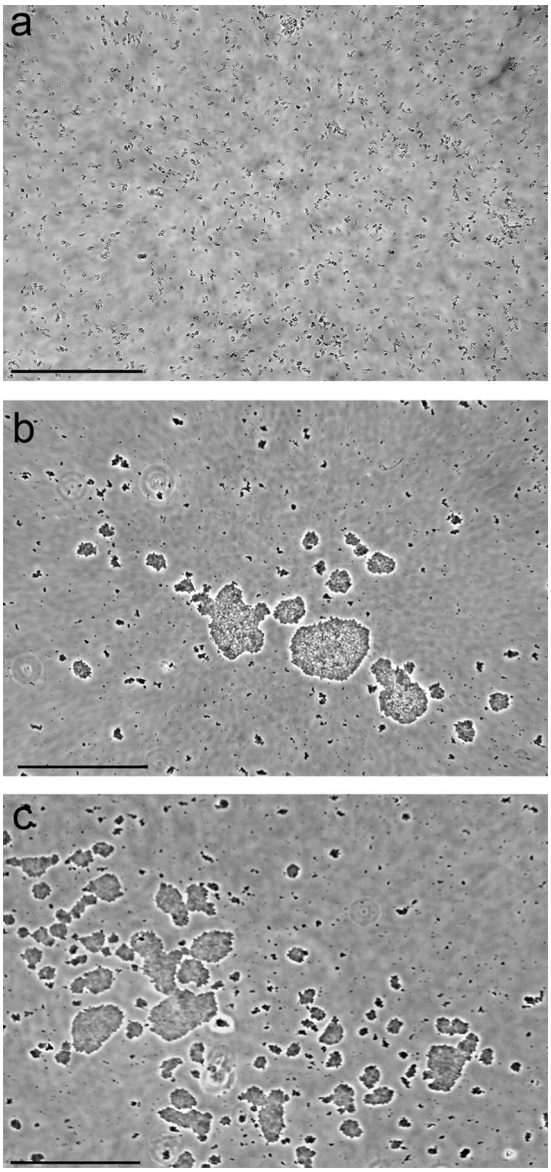

FIG. 1. Lectin agglutination of *H. pylori*. Photographs portray typical agglutination of *H. pylori* strain SS1 (10^8 cells/ml) observed under light microscopy after mixing bacteria for 5 s at room temperature with PBS (a), UEA-I at 20 μg/ml (b), or BS-I at 50 μg/ml (c). At these concentrations, the lectins produced aggregates of similar size. Bar, 100 μm.

conditions for 3 days at 37°C. Colonies were counted and CFU per gram of intestinal tissue calculated.

Immunization experiments. For the *H. pylori* vaccination/challenge experiment, mice ($n = 8$) were dosed orogastrically with 200 μl of either (i) PBS, (ii) 10^7 formalin-fixed *H. pylori* SS1 bacteria, (iii) 10^7 formalin-fixed *H. pylori* SS1 bacteria agglutinated by 20 μg UEA-I, (iv) 10^7 formalin-fixed bacteria plus 10 μg of cholera toxin (CT) (Sigma), or (v) 100 μg of *H. pylori* lysate or 10^7 formalin-fixed *H. pylori* bacteria plus 10 μg CT. Mice received two vaccinations spaced by 3 weeks, and then 4 weeks after the second immunization, mice were challenged orally with 10^7 live bacteria. Four weeks after challenge, the stomach was removed for the determination of bacterial load by colony-forming assay.

For the *C. jejuni* vaccination/challenge experiment, mice ($n = 8$) were dosed orogastrically with 200 μl of either (i) PBS, (ii) 10^8 formalin-fixed *C. jejuni* 81-176 bacteria, or (iii) 10^8 formalin-fixed *C. jejuni* 81-176 bacteria agglutinated by 100 μg UEA-I. Mice received two vaccinations spaced by 3 weeks, and then 4 weeks after the second immunization, mice were challenged orally with 10^7 live bacteria. One week after challenge, the intestines were removed for the determination of bacterial load by colony-forming assay.

Histological assessment of gastritis. Stomach halves were fixed in 10% neutral buffered formalin and embedded in paraffin, and 4-μm-thick sections were cut, stained with hematoxylin and eosin, and scored blinded under light microscopy. Inflammation was assessed in two separate gastric tissue sections for each animal, using three parameters. First, cellular infiltration was graded from 0 to 6 as follows: 0, none; 1, mild multifocal; 2, mild widespread or moderate multifocal; 3, mild widespread and moderate multifocal or severe multifocal; 4, moderate widespread; 5, moderate widespread and severe multifocal; and 6, severe widespread. As mice do not develop the classical gastric atrophy seen in *H. pylori*-infected humans, two surrogate markers were also used: "mucus metaplasia" (large, pale, globular cells in the corpus) and "functional atrophy" (loss of parietal cells). Metaplasia and atrophy were graded from 0 to 3 (absent, mild, moderate, and severe, respectively).

Statistical analysis. Statistical analyses were performed using SPSS software, version 16.0. The significance of differences between bacterial colonization and antibody levels was determined using log-transformed data by one-way analysis of variance (ANOVA) with Dunnett's post hoc analysis. For significance of histological grading scores, data were compared by nonparametric Mann-Whitney analysis.

RESULTS

Oral delivery of UEA-I-agglutinated live *Helicobacter pylori*. Our initial studies investigating the potential immunological and protective effects of M-cell targeting of a whole pathogenic bacterium used the *H. pylori* mouse model. It was shown previously that lipopolysaccharides from only some strains of *H. pylori* (but including the mouse-colonizing strain SS1) express fucose and are agglutinated by UEA-I (19, 26). To perform this study, we identified two mouse-colonizing strains of *H. pylori* with disparate UEA-I binding profiles. Using a range of lectin dilutions (1 to 500 μg/ml), we confirmed that while UEA-I potently agglutinated whole live *H. pylori* strain SS1 at the lowest dose tested (1 μg/ml), it did not agglutinate strain 11637, even at 500 μg/ml (Table 1). In contrast, both strains were similarly agglutinated by the lectin BS-I, which has specificity for glucose- or *N*-acetylglucosamine-containing saccharides.

This provided us with an ideal model for assessing the immunological and protective impacts of the targeted delivery of agglutinated bacteria to gastrointestinal M cells. C57BL/6 mice were orally dosed twice, 3 weeks apart, with viable *H. pylori* SS1 agglutinated with UEA-I, and then sera and intestinal scrapings were collected for analysis of the induced antibody response. *H. pylori*-specific IgG and IgA titers in both the sera and intestinal scrapings of mice that received UEA-I-agglutinated *H. pylori* SS1 were significantly enhanced compared to those in mice that received SS1 without lectin (Fig. 2). Some of these enhanced responses were substantial; for example, delivery of UEA-I-agglutinated *H. pylori* induced a 3-log-unit increase in specific serum IgG levels (Fig. 2a).

TABLE 1. Lectin-mediated agglutination of live *Helicobacter pylori*

Lectin	Specificity[a]	Minimum lectin concn (μg/ml) resulting in agglutination of *H. pylori*[b]:	
		SS1	NCTC 11637
None (PBS)		—	—
BS-I	α-Gal/α-GalNAc	50	100
UEA-I	Fucα1,2Galβ1,4GlcNAc	1	—

[a] Fuc, fucose; Gal, galactose; GalNAc, *N*-acetylgalactosamine; GlcNAC, *N*-acetylglucosamine.

[b] *H. pylori* (10^8 cells/ml) was incubated with lectins at 500, 100, 50, 25, 12.5, 5, 2.5, and 1 μg/ml and agglutination observed by light microscopy. PBS was used as a negative control. —, no agglutination at highest tested concentration (500 μg/ml).

This enhanced mucosal and systemic humoral response was not related to a nonspecific uptake of particulate bacterial aggregates, as no increase in antibody levels was present in mice dosed with *H. pylori* SS1 agglutinated with a different lectin, BS-I (Fig. 2a to d), which does not bind to M cells (30). The increased antibody response was also not due to any immunostimulatory activity of UEA-I, as codelivery with *H. pylori* 11637, which was not agglutinated by this lectin, did not result in enhanced antibody levels (Fig. 2a to d).

Vaccination of mice against *Helicobacter pylori* by oral delivery of UEA-I-agglutinated killed bacteria. The potent mucosal immune response induced by oral delivery of UEA-I-targeting *H. pylori* raised the possibility that this strategy may induce prophylactic protection against subsequent challenge. With this in mind, we next evaluated whether oral vaccination with UEA-I-agglutinated killed *H. pylori* would be as effective at inducing an elevated systemic and mucosal antibody response as live bacteria. As we have previously found that the host genetic background can influence the effectiveness of vaccine-induced protection against *H. pylori* (33), for this study BALB/c mice were used to confirm that the observed benefits of UEA-I targeting were maintained in recipients with different backgrounds.

After confirming that formalin-fixed *H. pylori* SS1 organisms were agglutinated by UEA-I to the same degree as live bacteria but did not colonize mice (data not shown), we compared the specific antibody responses induced by oral delivery of UEA-I-agglutinated formalin-fixed or live *H. pylori* SS1. UEA-I targeting of live agglutinated *H. pylori* induced greatly elevated levels of specific serum and intestinal antibodies in BALB/c mice (Fig. 3), as shown above for C57BL/6 mice, demonstrating that this approach was effective for different recipient backgrounds. Importantly, increased antibody levels were also induced when UEA-I-agglutinated formalin-fixed bacteria were used, with the response equivalent to that induced by UEA-I-agglutinated live bacteria (Fig. 3). Thus, live bacteria were not required for the elevated antibody response that occurred with UEA-I-targeted delivery. Further, we found that oral delivery of *H. pylori* SS1 lysate mixed with UEA-I did not induce an elevated antibody response, indicating that while live bacteria

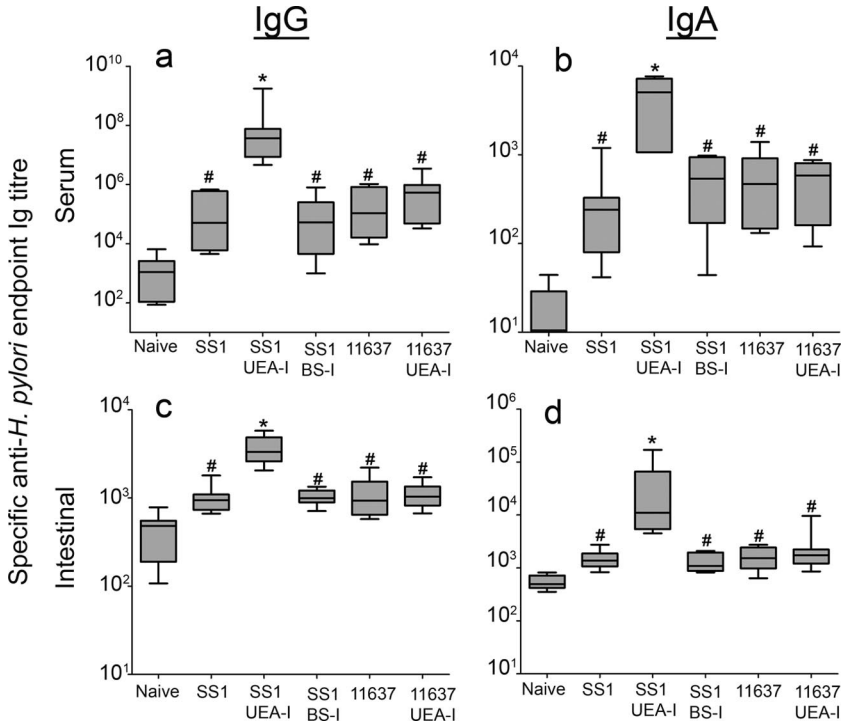

FIG. 2. Mucosal and systemic antibody responses induced by oral delivery of UEA-I-agglutinated live *H. pylori*. C57BL/6 mice ($n = 8$) were orally dosed twice with live *H. pylori* (SS1 or 11637) mixed with either PBS or the lectin BS-I or UEA-I. One week after the second dose, sera and intestinal scrapings were collected and specific anti-*H. pylori* antibody end point titers determined by ELISA. Box plots present the median antibody titers (horizontal bar), the interquartile range (boxed region), and the 10th and 90th percentile values (error bars). All treated groups had significantly elevated levels of anti-*H. pylori* IgG and IgA in their sera and intestines compared with the naïve group (#, $P < 0.05$ by ANOVA). Oral delivery of live *H. pylori* agglutinated with UEA-I lectin significantly increased IgG and IgA antibody titers compared with all other groups (*, $P < 0.005$ by ANOVA).

are not required, intact bacteria are essential for the observed potent mucosal and systemic immune response.

We then examined whether the increased mucosal immune response induced by UEA-I targeting of fixed bacteria would translate to improved protection against subsequent live challenge. Mice that were orally dosed with formalin-fixed *H. pylori* SS1 prior to homologous challenge with live bacteria had reduced colonization compared to unvaccinated controls (Fig. 4). However, this protection was significantly improved when the bacteria were agglutinated with UEA-I (Fig. 4). Protection induced by vaccination with fixed *H. pylori* agglutinated with UEA-I was equivalent to that resulting from oral delivery of fixed bacteria adjuvanted with CT as well as the gold standard comparator, bacterial lysate plus CT (Fig. 4). Vaccination against *Helicobacter* is commonly accompanied by a postimmunization gastritis, where gastritis in vaccinated/challenged animals is more severe than that induced by infection alone (12, 32). We therefore assessed the degree of inflammation in these mice at 4 weeks after bacterial challenge. Protection induced by vaccination with *H. pylori* plus CT was accompanied by a small but significant increase in cellular infiltrate into the gastric mucosa (Table 2). However, vaccination with UEA-I-agglutinated bacteria prior to live *H. pylori* challenge had no effect on gastritis, demonstrating that such vaccinations can induce protective immunity without exacerbating inflammation.

UEA-I-targeted vaccination of mice against the intestinal pathogen *Campylobacter jejuni*. While mucosal targeting of fixed *H. pylori* induced markedly elevated mucosal and systemic antibodies, only partial protection was achieved following live challenge. We theorized that the incomplete protection may be due to the fact that protective immunity against *H. pylori* following vaccination is not mediated by antibodies (4, 13, 34). We therefore extended this study by selecting a bacterial infection (*C. jejuni* infection) where humoral immunity is believed to be protective (2, 25, 36). In mice, *C. jejuni* colonizes the mouse intestine for a period of 2 to 3 weeks before being naturally cleared (36). We therefore assessed whether mucosal targeting would provide protection against this pathogen.

First, we identified that the mouse-colonizing *C. jejuni* strain 81-176 was agglutinated by UEA-I (at 100 μg/ml of lectin [not shown]). As for *H. pylori*, delivery of UEA-I-agglutinated live *C. jejuni* induced a significant elevation in both serum and intestinal antibody levels (Fig. 5). Colonization levels in BALB/c mice receiving UEA-I-agglutinated live *C. jejuni* were reduced relative to that in nontargeted controls at 1 week postchallenge, although all mice were infected (Fig. 6). As expected, at 2 weeks postdelivery of live *C. jejuni*, colonization levels were naturally falling. However, clearance was accelerated in mice that received UEA-I-targeted live *C. jejuni*, and three of these mice had no detectable bacteria (Fig. 6).

Finally, we examined the protective effect of vaccinating

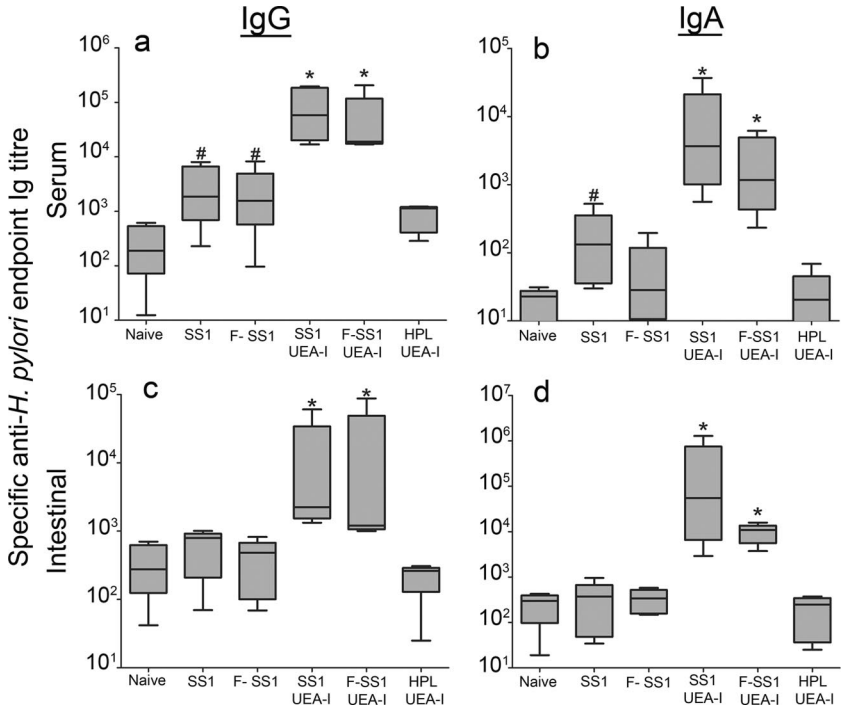

FIG. 3. Mucosal and systemic antibody responses induced by oral delivery of UEA-I-agglutinated, formalin-fixed *H. pylori*. BALB/c mice (n = 5) were orally dosed twice with UEA-I-agglutinated live *H. pylori* (SS1 UEA-I), formalin-fixed *H. pylori* (F-SS1 UEA-I), or *H. pylori* lysate (HpL). Control groups received the same bacteria without lectin (SS1 and F-SS1, respectively) or were left untreated (naïve). One week after the second dose, sera and intestinal scrapings were collected and specific anti-*H. pylori* antibody end point titers determined by ELISA. Box plots present the median antibody titers (horizontal bar), the interquartile range (boxed region), and the 10th and 90th percentile values (error bars). *, oral delivery of UEA-I-agglutinated, fixed bacteria induced mucosal and systemic antibody levels equivalent to those for UEA-I-agglutinated live bacteria but significantly greater than those for all other groups ($P < 0.01$ by ANOVA). #, significantly greater than untreated control ($P < 0.04$ by ANOVA).

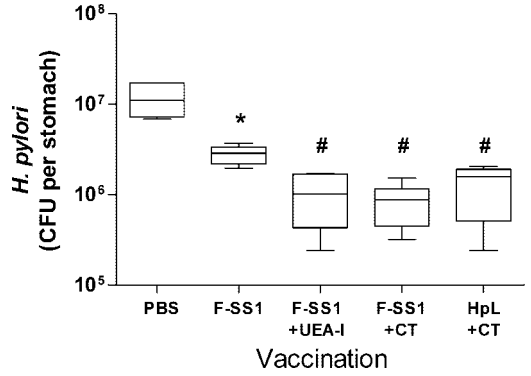

FIG. 4. Protective immunity against *H. pylori* induced by oral delivery of UEA-I-agglutinated bacteria. BALB/c mice (n = 8) were orally dosed twice with formalin-fixed *H. pylori* (F-SS1) either alone or agglutinated with UEA-I. Negative controls received PBS, while positive controls were vaccinated with either *H. pylori* lysate (HpL) plus CT or F-SS1 plus CT. Four weeks after the second dose, all mice were challenged with *H. pylori* SS1, and the bacterial burden was determined a further 4 weeks later by colony-forming assay. *H. pylori* colonization levels were calculated as CFU per stomach. Box plots present the median level of colonization (horizontal bar), the interquartile range (boxed region), and the 10th and 90th percentile values (error bars). Data shown are from a single experiment and are representative of two separate experiments. *, significantly reduced colonization compared with the negative control ($P < 0.001$ by ANOVA). #, significantly reduced colonization compared with both the unvaccinated control group and the group receiving formalin-fixed bacteria alone ($P < 0.01$ by ANOVA).

mice with formalin-fixed *C. jejuni*, with or without UEA-I-mediated targeting. Oral vaccination of mice with formalin-fixed *C. jejuni* produced some protection against subsequent challenge with live bacteria, resulting in a reduced bacterial burden at 1 week postchallenge (Fig. 7). However, all these mice were still infected. In contrast, 7/8 mice that were immunized with UEA-I-agglutinated *C. jejuni* were completely protected against live challenge, with no detectable bacteria at 1 week after challenge (Fig. 7). The single infected mouse had only a very low-level bacterial burden. Thus, UEA-I-mediated targeting of formalin-fixed *C. jejuni* improved protection resulting from oral delivery of fixed bacteria alone and was com-

TABLE 2. Vaccination of BALB/c mice with UEA-I-agglutinated *H. pylori* prior to live bacterial challenge does not induce postimmunization gastritis

Vaccination[a]	Median grade of pathology (interquartile range)		
	Cellular infiltration	Mucus metaplasia	Atrophy
None	0 (0–0.5)	0 (0–0)	0 (0–0)
F-SS1 plus UEA-I	0 (0–0)	0 (0–0)	0 (0–0)
F-SS1 plus CT	1 (0–2)[b]	0 (0–0)	0 (0–0)

[a] Groups of BALB/c mice (n = 8) were vaccinated twice orally prior to challenge of all mice with *H. pylori* SS1. Four weeks after the second vaccination, all mice were challenged with *H. pylori* SS1, and gastric pathology was assessed a further 4 weeks later. F-SS1, formalin-fixed *H. pylori* strain SS1.

[b] Significantly greater than value for unvaccinated but infected controls ($P < 0.05$ by Mann-Whitney test).

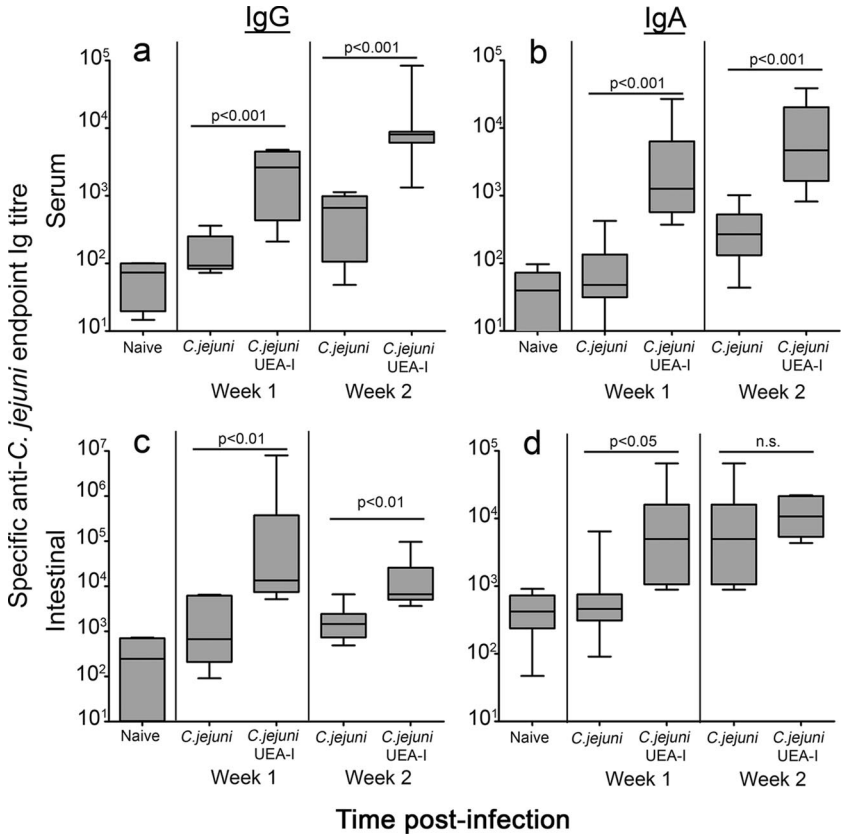

FIG. 5. Mucosal and systemic antibody responses induced by oral delivery of UEA-I-agglutinated live *C. jejuni*. BALB/c mice ($n = 16$) were orally dosed with live *C. jejuni* 81-176, either mixed with PBS or agglutinated by UEA-I. One and two weeks later, sera and intestinal scrapings were collected from eight mice and specific anti-*C. jejuni* antibody end point titers determined by ELISA. Box plots present the median antibody titers (horizontal bar), the interquartile range (boxed region), and the 10th and 90th percentile values (error bars). UEA-I-mediated agglutination significantly elevated the levels of anti-*C. jejuni* IgG and IgA in sera and intestines compared with the group receiving bacteria alone as determined by ANOVA.

pletely protective in the majority of immunized animals. None of the animals had evidence of inflammation in their intestines (data not shown).

DISCUSSION

One of the key issues preventing the development of killed or subunit mucosal vaccines is the lack of mucosal adjuvants that are both safe and effective. Immunostimulatory mucosal adjuvants typically used for effective oral vaccination in rodent models, such as CT or heat-labile toxin from *Escherichia coli* (LT), are considered too toxic for use in humans, resulting in diarrhea when given orally (24) and being associated with Bells' palsy when delivered nasally (10). Thus, strategies that can circumvent the requirement for the addition of exogenous immunostimulatory adjuvants may be of considerable benefit.

We have demonstrated here that oral delivery of formalin-fixed bacteria agglutinated with the M-cell-targeting lectin UEA-I typically induces a 100-fold increase in both the serum and intestinal antibody responses compared with nontargeting vaccination. The elevated mucosal antibody response induced by UEA-I required the presence of whole bacteria, as it did not occur in mice immunized with lysate plus UEA-I. This response was not mediated purely by the formation of particulate clumps, as agglutination of the same bacteria with a different lectin (BS-I) with an alternative glycan specificity had no effect. Finally, the response was not due to any nonspecific immunostimulatory effects of this lectin on the host immune response, as when UEA-I was codelivered with a strain of *H. pylori* that it did not agglutinate or with lysate of an agglutinating strain, no enhanced immune response resulted. This is quite different from the case for mistletoe lectins, which have also been examined as oral adjuvants. These were demonstrated to stimulate the host immune system and induce a response against a codelivered but nonassociated antigen (22).

The immune response induced by M-cell targeting also afforded protection against pathogen challenge, albeit with variable effectiveness. When used as a prophylactic vaccine against *H. pylori*, oral delivery of UEA-I-agglutinated, fixed bacteria enhanced the protective response against subsequent challenge. Raghavan et al. have previously shown that oral delivery of formalin-fixed *H. pylori* SS1 can induce some protection against homologous challenge (29). Our data extend their finding by demonstrating that this protection can be improved by the use of UEA-I-agglutinated fixed bacteria. Notably, this protective immunity was equivalent to that induced by fixed bacteria plus CT, demonstrating that UEA-I-mediated targeting could replace the requirement for this potent muco-

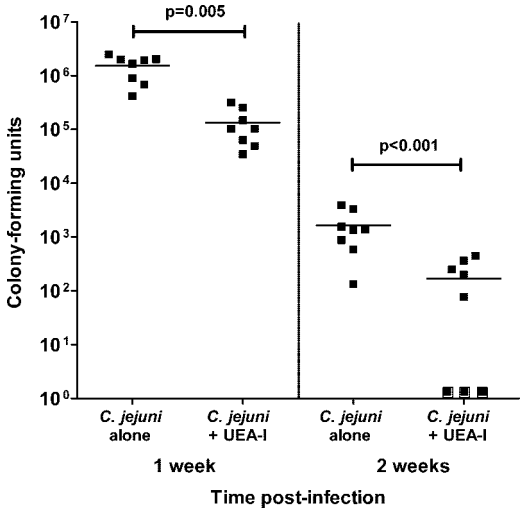

FIG. 6. Intestinal colonization following oral delivery of lectin-agglutinated *C. jejuni*. BALB/c mice ($n = 16$) were orally dosed with live *C. jejuni* 81-176, either mixed with PBS or agglutinated by UEA-I. One and two weeks later, intestines were removed from eight mice and bacterial colonization levels determined by colony-forming assay. Colonization levels for individual mice are shown, with the median level of colonization for each group (horizontal bar). Delivery of UEA-I-agglutinated *C. jejuni* significantly reduced colonization levels at both time points compared with nonagglutinated controls as determined by ANOVA.

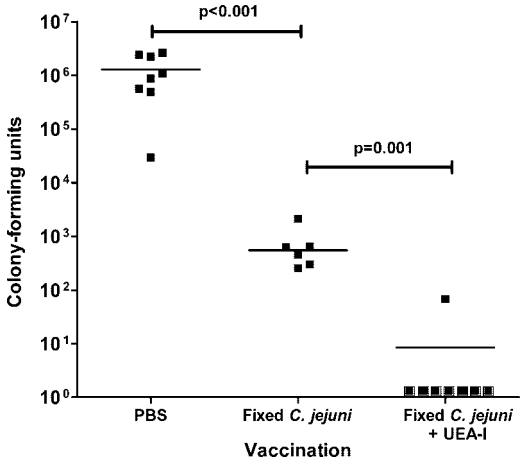

FIG. 7. Induction of protective immunity against *C. jejuni* by oral vaccination with UEA-I-agglutinated bacteria. BALB/c mice ($n = 8$) were orally vaccinated twice with formalin-fixed *C. jejuni* 81-176 either alone or agglutinated with UEA-I. Negative controls received PBS. Four weeks after the second vaccination, all mice were challenged with *C. jejuni* 81-176, and the bacterial burden was determined a further 4 weeks later by colony-forming assay. Colonization levels for individual mice are shown, with the median level of colonization for each group (horizontal bar). Oral vaccination with fixed *C. jejuni* induced significant protection against subsequent live challenge compared with unvaccinated controls as determined by ANOVA. Protection was significantly increased when vaccinating bacteria were agglutinated with UEA-I (as determined by ANOVA).

sal adjuvant. The protection induced against *H. pylori* by this strategy was insufficient to clear the infection, which is consistent with the idea that, despite induction of *H. pylori*-specific antibodies, protective immunity against this pathogen is antibody independent (4, 13, 34). As it has been shown that vaccine-induced protection against *H. pylori* requires $CD4^+$ T cells (13, 28), the induction of protection against this pathogen by M-cell targeting indicates that this approach induced an effective T-cell response. In contrast, clearance of primary *C. jejuni* infection of mice is believed to be antibody mediated (36). Using *C. jejuni* mouse models, oral vaccination with fixed whole *Campylobacter* has been shown to induce incomplete protection against live challenge (3). When we vaccinated mice with UEA-I-agglutinated killed *C. jejuni*, sterilizing immunity was achieved in the majority of animals.

This demonstrates that appropriate mucosal delivery of whole killed bacteria can induce complete protection against certain pathogens without a requirement for immunostimulatory adjuvants. Typical mucosal adjuvants such as CT and LT have direct activity on host immunity (1) and therefore do not require direct association with target antigen. Our finding that UEA-I activity required direct association with whole bacteria argues that it functions purely as a delivery system, targeting the bacteria to mucosal immune cells. Injection of a recombinant *H. pylori* antigen into the Peyer's patches of mice gave protection against subsequent challenge when delivered with incomplete Freund's adjuvant (11). However, we have now demonstrated that by using a strategy that targets intestinal M cells, we can remove the requirement for potentially toxic adjuvants.

The central aim of this study was to explore the potential for targeted delivery of bacterial vaccines without toxic adjuvants. We therefore did not examine whether addition of a mucosal adjuvant such as CT or LT would further increase this protection. However, when effective mucosal adjuvants that are safe for use in humans become available, it would be very interesting to examine whether their addition would further boost the levels of mucosal antibody response and protection induced by mucosal targeting. This would be particularly relevant for *H. pylori*, where UEA-I-targeted delivery is clearly insufficient to generate complete protection, although it is possible that this could be achieved in combination with an appropriate safe adjuvant. There is clearly considerable potential for this method as a strategy against pathogens such as *C. jejuni*, although in this specific case other factors need to be considered, such as the concern that these bacteria may be involved in the development of Guillain-Barré syndrome and that a whole *C. jejuni* vaccine could possibly induce this disease (37).

In summary, this study provides proof of principle, using two different model systems, that M-cell targeting of whole pathogenic bacteria can enhance mucosal and systemic antibody responses as well as generate protective immunity against challenge. Some pathogens, such as poliovirus and *Salmonella enterica* serovar Typhi, naturally bind to M cells during infection, resulting in investigations of the potential use of these organisms as delivery systems. Moreover, it has been proposed that lectins could be used to deliver live attenuated pathogenic bacteria (21). Such an approach has considerable merit but requires pathogen attenuation and involves all of the ongoing concerns associated with the use of live vaccines. Here we have shown that a far simpler and

potentially safer strategy is feasible, by which the requirement for live pathogens is removed.

It is possible that agglutination is not essential and that M-cell delivery of individual whole bacteria may induce the same response. Due to the multivalent nature of UEA-I lectin, this cannot be examined readily in the model used in this study. However, this could potentially be achieved by engineering bacteria to express an M-cell binding receptor on their surfaces. For translation to human use, it may actually be preferable to deliver individual bacteria to M cells rather than bacterial clumps, as this would facilitate manufacture of a consistent and highly reproducible product.

While UEA-I lectin targets murine M cells, it does not bind specifically to human M cells (30), so this particular lectin cannot be used for human vaccine delivery. However, human M cells express their own specific carbohydrate moieties, for example, preferentially displaying sialyl Lewis A (16). Alternative lectin delivery systems could therefore be potentially exploited to develop an immunostimulatory adjuvant-free human vaccine. Further, it was recently demonstrated that it is possible to generate specific monoclonal antibodies to the mouse M cell (27). Hence, while challenges remain, current technological developments may well allow a strategy for human M-cell delivery to develop a new generation of effective killed bacterial vaccines that can circumvent the problems associated with toxic adjuvants.

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