Escherichia coli O157: H7 colonization in cattle following systemic and mucosal immunization with purified H7 flagellin

Citation for published version:

Digital Object Identifier (DOI):
10.1128/IAI.01452-07

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published In:
Infection and Immunity

General rights
Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy
The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.
**Enterohemorrhagic Escherichia coli (EHEC)** is a zoonotic pathogen of worldwide importance, causing severe diarrhea (hemorrhagic colitis) and, in a small percentage of cases, hemolytic-uremic syndrome in humans. Ruminants are an important reservoir of EHEC, and human infections are frequently associated with direct or indirect contact with ruminant feces, particularly those derived from cattle (16, 26, 34, 36). Coincidentally, strategies to reduce the carriage of EHEC in ruminants are predicted to lower the incidence of human disease (reviewed in reference 36), and stochastic simulation models predict that cattle are a key control point to reduce EHEC infections in humans (22).

An attractive strategy to reduce EHEC colonization in cattle is by vaccination. A number of EHEC vaccines have been evaluated in cattle and have primarily focused on immunization with bacterial proteins encoded by genes located within the locus of enterocyte effacement (LEE) that are known to interact with bacterial proteins encoded by genes located within the virulence factor for adherence (encoded by *inta*). Another protein implicated in bovine intestinal colonization (37), and formalin-inactivated EHEC bacterin have been evaluated (40). However, with the exception of immunization with the secreted protein preparation, which resulted in a reduction in bacterial shedding in calves following experimental challenge and reduced prevalence of EHEC in a small but not a large-scale field trial (39), all EHEC vaccines evaluated have had no effect on subsequent EHEC colonization in cattle.

The reasons for the lack of success of EHEC vaccines to date could be twofold. First, it may indicate that additional vaccine targets are required. Second, as EHEC is nonpathogenic in cattle and is restricted to the intestinal epithelium and gut lumen (reviewed in reference 36), intestinal mucosal antibodies to bacterial epithelial adhesins are likely to represent key effectors for reduction of intestinal colonization in cattle. Indeed, passive immunization of piglets with colostrum containing intimin-specific antibodies has been shown to confer resistance to subsequent EHEC challenge, supporting a role for mucosal antibodies in reducing bacterial colonization in vivo.
(10). However, in previous EHEC vaccine trials, antigen-specific mucosal antibody responses postimmunization either were not measured (35) or were poor (14, 40). Therefore, the lack of vaccine efficacy may in part be due to poor levels of antigen-specific antibody at intestinal mucosal surfaces.

In North America and Europe, the predominant EHEC serotype affecting humans is O157:H7 (1, 2). The principal site of E. coli O157:H7 colonization in cattle is the terminal rectum (31). Work in our laboratory has indicated that H7 flagella play an important role in initial binding of E. coli O157:H7 to bovine primary rectal epithelial cells in vitro (27), and Erdem et al. have also recently demonstrated that the presence of H7 flagella is important in bacterial adherence to bovine intestinal tissue explants (15). Furthermore, H7 flagella have been shown to play a role in E. coli O157:H7 colonization of chickens in vivo (3), and flagella of a number of other bacteria, including Clostridium difficile (38), Pseudomonas aeruginosa (25), and enteropathogenic E. coli (17), have been demonstrated to act as epithelial adhesins. Together, these observations suggest that H7 flagella may represent an additional target for E. coli O157:H7 vaccination in cattle.

In this study, we evaluated the effects of systemic and mucosal immunization with purified H7 flagellin, the main structural component of H7 flagella, on subsequent E. coli O157:H7 colonization in cattle. In an attempt to induce high levels of mucosal antibodies at the principle site of colonization of E. coli O157:H7 in cattle, i.e., the terminal rectum, mucosal immunizations with either H7 flagellin alone or H7 incorporated into poly(3-lactide-co-glycolide) (PLG) microparticles (PLG: H7) were administered onto the rectal mucosa, which in cattle possesses characteristics of an immune inductive site (28). Furthermore, we performed a detailed analysis of H7-specific mucosal antibody levels following immunizations using previously validated mucosal sampling protocols (29).

MATERIALS AND METHODS

Purification of E. coli O157:H7 flagellin and encapsulation into PLG microparticles. Flagellin (H7) was isolated from E. coli O157:H7 (stx mutant) strain Zap984, a LEE4 deletion mutant derived from strain Zap198 (32) by acid dissociation, pH reassociation, and ammonium sulfate precipitation as previously described (19). This protocol results in the purification of flagellin monomers, which spontaneously repolymerize into flagellar filament at neutral pH. Purity was verified using polyacrylamide gel electrophoresis, followed by Simply Blue staining (Invitrogen, San Diego, CA) and by Western blotting.

PLG:H7 microparticles were prepared using the water/oil/water solvent evaporation technique as previously described (20). Briefly, 100 µl of H7 flagellin at 10 mg/ml in distilled water was emulsified with 2 ml of a 5% solution of PLG (Sigma-Aldrich, St. Louis, MO) in dichloromethane at 16,000 rpm for 2 min using a T25 Basic Ultra-Turrax homogenizer (IKA-Werke, Staufen, Germany) to produce a water-in-oil emulsion. The emulsion was subsequently homogenized with 20 ml of 5% polyvinyl alcohol solution at 19,000 rpm for 2 min to produce a stable water/oil/water emulsion. The secondary emulsion was stirred for 12 to 18 h at room temperature (RT) and pressure to allow solvent evaporation to occur and subsequent microparticle preparation. The microparticles were collected by centrifugation, washed three times with distilled water, and lyophilized. This procedure was repeated on five separate occasions, and the resultant microparticles were pooled for subsequent immunizations.

The protein content and ratio of surface-bound to encapsulated protein of each batch of microparticles was assessed by using a bicinchoninic protein assay kit (Pierce, Rockford, IL) following removal of surface protein from a known weight of microparticles with 2% sodium dodecyl sulfate and subsequent dissolution of the particles in 0.5 M NaOH to release encapsulated protein. The released protein was subjected to Western blotting. The particle size and the number of particles per mg PLG:H7 was estimated using a Z1 Coulter particle counter (Beckman Coulter Inc., Fullerton, CA).

Western blotting. Samples of H7 flagellin or digested PLG:H7 microparticles were separated by polyacrylamide gel electrophoresis using NuPAGE 4–12% Bis-Tris gels (Invitrogen) under reducing conditions and transferred to nitrocellulose membranes (pore size, 0.2 µm) using the Xcell II blotting system (Invitrogen) according to the manufacturer’s instructions. The membranes were subsequently washed in phosphate-buffered saline (PBS) containing 0.5 M NaCl and 0.5% (vol/vol) Tween 80 for 1 h at RT. The blots were incubated for 1 h at RT with rabbit polyclonal anti-H7 antibody (Mast-Assure, Bootele, United Kingdom) diluted 1:500 in PBS containing 0.5 M NaCl and 0.5% (vol/vol) Tween 80, followed by incubation for 1 h at RT with a 1:2,000 dilution of goat anti-rabbit polyclonal antibody conjugated to horseradish peroxidase (HRP) (Dakocytomation, Ely, United Kingdom). Controls included omission of the primary antibody and replacement of primary antibody with normal rabbit serum. HRP was detected after the final washing by incubating the blots with ECL Plus reagent (GE Healthcare, Little Chalfont, United Kingdom).

Immunization protocol and oral bacterial challenge. Immunizations and oral bacterial challenges were performed at the Morehead Research Institute under Home Office license 60/3179. Ethical approval was obtained from the Morehead Research Institute Animal Experiments Committee. Four groups of eight conventionally reared male Holstein-Friesian calves were immunized on three separate occasions at 2-week intervals as follows: group 1 received 60 µg H7 flagellin plus 5 mg Qul A (Brenntag Biosector, Frederikskund, Denmark) in 2 ml PBS intramuscularly (i.m.); group 2 received 60 µg H7 flagellin in 1 ml PBS administered intrarectally using a paste inserted approximately 4 cm beyond the anus; group 3 received 60 µg H7 flagellin incorporated into PLG microparticles (PLG: H7) administered intrarectally; group 4 received no immunizations (nonvaccinated control group). The average age of the calves at the time of the first immunization was 9 ± 2 weeks, and fecal samples obtained from each calf prior to immunization were confirmed to be negative for E. coli O157:H7 by immunomagnetic separation performed according to the manufacturer’s instructions (Dynabeads anti-E. coli O157; Invitrogen).

Ten days after the last immunization, the calves were orally challenged with 105 CFU naldixic acid-resistant E. coli O157:H7 (stx mutant) strain Zap198, and viable E. coli O157:H7 bacteria per gram of surface feces were enumerated once daily for 3 weeks postchallenge by plating them onto sorbitol MacConkey agar plates containing 15 µg/ml naldixic acid (Oxoid, Basingstoke, United Kingdom) as previously described (31). To estimate the total bacterial fecal shedding, daily bacterial counts were plotted versus time for each calf, and the area under the shedding curve (AUC) was calculated. In addition, bacterial uptake rates (as defined by an increasing fecal bacterial count from 3 days postchallenge) were recorded. Serum, nasal secretions, and rectal swabs were collected as described previously (29) 4 days prior to the first immunization, 1 week after each immunization, and 2 weeks after oral bacterial challenge. The calves were euthanized 3 weeks after bacterial challenge, and abomasal and small-intestinal swabs were collected, in addition to serum, nasal secretions, and rectal swabs.

Quantification of anti-H7 flagellin and anti-O157 LPS antibodies. H7 flagellin-specific immunoglobulin A (IgA) and IgG antibodies were quantified in serum, nasal secretions, and intestinal-swab samples by indirect enzyme-linked immunosorbent assay (ELISA) as described previously (29). To measure anti-O157 lipopolysaccharide (LPS) antibodies, plates were coated with 0.1 µg/well O157 LPS conjugated to polymyxin as previously described (9) and subsequently processed identically to the H7 ELISAs. Optimum sample dilutions were determined following serial dilution of representatives from each to ensure that the color reaction product at an optical density at 492 nm (OD492) for the samples was on the linear part of the curve. Samples were diluted 1:1,000, 1:10, and 1:2.5 for serum, nasal secretions, and intestinal-swab samples, respectively, for IgG ELISAs and 1:10, 1:100, and 1:2.5 for serum, nasal secretions, and intestinal-swab samples, respectively, for IgG ELISAs. ODs obtained for intestinal-swab samples were normalized to the total IgA, measured using a sandwich ELISA obtained from Bethyl Laboratories Inc. (Montgomery, TX), and interplate variation was normalized to a positive control. Western blotting was also performed using ECL Plus reagent (GE Healthcare) on selected preimmunization, postimmunization, or post-bacterial-challenge serum, nasal secretions, and rectal-swab samples from each immunization group to confirm the specificity of the antibody responses measured by ELISA.

Quantification of anti-H7 flagellin IgA and IgG. Next pre- and postimmunization naso-secretion samples from five i.m. H7-immunized calves were clarified by centrifugation at 11,000 X g for 5 min. Protein fractionation of 0.5 ml nasal secretion was carried out by gel filtration chromatography using a Superdex 200 10/300 column and the AKTA fast-protein liquid chromatography system (GE Healthcare Biosciences AB, Uppsala, Sweden). The column was equilibrated
RESULTS

Characterization of PLG:H7 microparticles. Approximately 94% of the microparticles were <3 μm in diameter, with a further 5% of the particles between 3 and 5 μm in diameter. The efficiency of encapsulation of H7 flagellin into microparticles ranged between 35 and 40%. Of the total encapsulated protein, it was estimated that 24 to 35% was surface bound, with the remaining 65 to 76% internalized into the microparticles. The number of particles per mg of PLG:H7 of the pooled microparticles from all batches was estimated to be approximately 8 × 10^10 particles/mg PLG:H7, and each mg of PLG:H7 was equivalent to 6 μg H7. Digestion of microparticles released a single protein of approximately 60 kDa (Fig. 1A), which reacted strongly with commercial anti-H7 antibody following Western blotting (Fig. 1B). A band of approximately 120 kDa was also weakly reactive to commercial anti-H7 anti-

body. However, this band is commonly seen in our H7 flagellar preparations and has been previously identified as H7 flagellin by matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (27), suggesting that the band may represent H7 with posttranslational modifications or H7 dimers.

H7-specific antibody responses following immunization with H7 flagellin and subsequent oral bacterial challenge. Serum, nasal, and rectal H7-specific antibody responses are shown in Fig. 2. High titers of anti-H7 IgG and IgA were induced in both serum and nasal secretions following i.m. injection of H7, and IgA antibody levels reached peak levels after the second immunization, whereas IgG antibody levels continued to rise (Fig. 2A to D). In contrast, per-rectal immunization with either H7 or PLG:H7 failed to induce any significant serum or nasal H7-specific antibody response. Subsequent oral challenge with *E. coli* O157:H7 resulted in a small but significant increase in serum anti-H7 IgG within all immunized and control groups compared to postimmunization levels (*P* < 0.01 for all groups) (Fig. 2A). Bacterial challenge also resulted in a small but significant increase in nasal anti-H7 IgG (*P* < 0.05) (Fig. 2C) and a significant increase in nasal and serum IgA in animals from rectally immunized and control groups, but not from i.m.-immunized calves (*P* < 0.01 for both) (Fig. 2B and D). Anti-H7 IgG antibodies were detected in rectal-swab samples following i.m. immunization with H7, but not with any other immunization group (Fig. 2E). Rectal H7-specific IgA antibodies were induced following both i.m. vaccination and rectal immunization with H7 in PBS, and 1 week after the final immunization (day 42), the levels of anti-H7 IgA were similar in the two groups (Fig. 2F). In contrast, rectal immunization with PLG:H7 failed to induce any rectal anti-H7 IgA antibodies. Following bacterial challenge, a significant increase in rectal anti-H7 IgA, but not IgG, was observed in both rectally immunized groups and the nonvaccinated control group (*P* < 0.01 for all groups). No significant increase in H7-specific IgA or IgG was observed in i.m.-immunized calves in response to bacterial challenge.

Postmortem abomasal and small-intestinal H7-specific antibody levels are shown in Fig. 3. Significantly higher levels of anti-H7 IgA antibodies were found in the abomasum and small
intestines of i.m. immunized calves compared to rectally immunized and nonvaccinated calves (one-way ANOVA; main \( P \) values for both < 0.0001; significance at the 1% level using Tukey’s pairwise comparisons). No differences in abomasal or small-intestinal H7-specific IgA levels were observed between immunization groups.

Western blotting was performed on selected preimmunization, postimmunization, or post-bacterial-challenge samples from each immunization group. This confirmed that immuno-reactivity was primarily directed against H7 flagellin (data not shown).

**Correlation between H7- and LPS-specific antibody responses following oral bacterial challenge.** Oral bacterial challenge with *E. coli* O157:H7 resulted in an increase in H7-specific IgA antibodies in mucosal and serum samples from all groups, with the exception of the i.m. immunized group. To determine whether H7 responses following bacterial challenge in the i.m. immunized group were correlated with levels of...
bacterial colonization, anti-O157 LPS IgA antibodies (used as an indicator of *E. coli* O157:H7 mucosal immune responses) (30) were measured in serum, nasal secretions, and rectal-swab samples in i.m. immunized and nonvaccinated calves 12 days post-bacterial challenge. Correlations between anti-H7 and LPS IgA responses at day 12 postchallenge are shown in Table 1. In nonvaccinated calves, there was a significant positive correlation between anti-H7 and LPS antibody responses in serum, nasal secretions, and rectal-swab samples. In i.m. immunized calves, a significant positive correlation existed between H7 and LPS antibody levels in rectal swabs, but not in serum or nasal secretions.

**E. coli O157:H7 colonization following immunization with H7 flagellin.** Following immunization, oral bacterial challenge with *E. coli* O157:H7 resulted in successful colonization of three of eight calves in the i.m. immunized group compared to 100% of rectally immunized and six of eight nonvaccinated calves. Comparison of uptake rates between groups using Fisher’s exact test identified significant differences in uptake rates between the groups (*P* = 0.006), with the i.m. immunized group having a markedly lower uptake rate than the other groups.

Daily mean bacterial counts for colonized calves only are shown in Fig. 4A. There was an overall immunization times time interaction (*P* = 0.009) that was reflected in higher mean bacterial counts in the i.m. immunized group compared to rectally immunized and control groups on days 12 and 13. No differences in mean bacterial counts were identified between groups at other time points. Peak bacterial shedding occurred between days 10 and 14 in colonized calves from the i.m. immunized group, whereas peak shedding in colonized calves from rectally immunized and control groups occurred between days 5 and 8. To estimate total bacterial shedding, AUCs were calculated for each calf and are shown in Fig. 4B. No significant differences in AUCs were identified between immunization and control groups,

**TABLE 1. Correlation coefficients between anti-H7 and anti-O157 LPS IgA antibodies following oral challenge with *E. coli* O157:H7**

<table>
<thead>
<tr>
<th>Sample</th>
<th>i.m. H7-immunized group</th>
<th>Nonvaccinated group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>r</em></td>
<td><em>P</em> value</td>
</tr>
<tr>
<td>Serum</td>
<td>0.456</td>
<td>0.303</td>
</tr>
<tr>
<td>Nasal secretions</td>
<td>0.524</td>
<td>0.182</td>
</tr>
<tr>
<td>Rectal swabs</td>
<td>0.865</td>
<td>0.006</td>
</tr>
</tbody>
</table>

*Pearson’s correlation coefficient calculated using log-transformed data.*
indicating no differences in total bacterial shedding between these groups.

**Effects of anti-H7 IgA and IgG on intestinal epithelial binding of* E. coli* O157:H7 in vitro.** To determine if anti-H7 IgG and/or IgA isotypes are effective at inhibiting *E. coli* O157:H7 intestinal epithelial binding, wild-type *E. coli* O157:H7 or an isogenic aflagellar mutant strain was incubated with either anti-H7 IgG or IgA purified by gel filtration chromatography from nasal secretions derived from five i.m. immunized calves, and subsequent bacterial binding to bovine primary rectal epithelial cells was evaluated. Gel filtration fractions were analyzed for total IgA/IgG and H7-specific IgA/IgG. A representative graph of total IgA and IgG levels in fractions obtained from one calf is shown in Fig. 5A, and a graph of anti-H7 antibody levels in fractions from pre- and postimmunization nasal secretions from the same calf is shown in Fig. 5B. IgA was present in fractions 7 to 11 from all calves, with maximal total IgA present in fraction 9. The peak total IgG concentration was present in fraction 12, with small quantities of IgG present in fractions 7 to 9. Measurement of anti-H7 IgA and IgG antibodies in postimmunization fractions revealed maximal anti-H7 IgA and IgG antibodies in fractions 9 and 12, respectively. No anti-H7 IgA was detected in IgG-enriched fractions. However, some anti-H7 IgG activity was detected in IgA-enriched fractions. Minimal reactivity to anti-H7 IgA and IgG ELISAs was observed in fractions obtained from preimmune nasal secretions from the same calves. Anti-H7 IgM antibodies were also measured in all fractions and were not detected (data not shown).

The effects of anti-H7 IgA- or IgG-enriched fractions (9 and 12, respectively) on epithelial binding are shown in Fig. 5C and D. Incubation of wild-type *E. coli* O157:H7 with both anti-H7 IgA and IgG fractions resulted in a significant reduction in bacterial binding to bovine rectal epithelial cells compared to incubation with preimmune IgA and IgG fractions (Fig. 5C), with both isotypes inhibiting bacterial binding to similar de-
gree (one-way ANOVA; main $P$ value $< 0.001$; significance at the 1% level using Tukey’s pairwise comparisons). No significant differences in bacterial binding were observed following incubation of the isogenic aflagellar mutant strain with either antibody isotype (Fig. 5D), indicating that the observed inhibition of bacterial binding was a flagellin-specific effect.

**DISCUSSION**

Systemic immunization with H7 flagellin resulted in reduced colonization rates and delayed peak bacterial shedding in colonized calves by approximately 1 week. However, no effect on total bacterial shedding following systemic immunization was observed. In contrast, rectal immunization with either H7 alone or H7 incorporated into PLG microparticles had no effect on subsequent bacterial colonization or shedding. Alterations in bacterial shedding following systemic H7 immunization correlated with a widespread mucosal antibody response to H7 consisting of both IgG and IgA isotypes: anti-H7 IgG was detectable postmortem throughout the gastrointestinal tract, and anti-H7 IgA was detectable in both rectal-swab samples and nasal secretions, suggesting that i.m. immunization resulted in induction of an IgA response at multiple mucosal sites. The lack of any effect on bacterial colonization following rectal immunization with H7 in PBS occurred despite induction of a local (rectal) IgA response. Immunization with PLG:H7 failed to induce any detectable anti-H7 antibodies, possibly due to either insufficient uptake of antigen at the rectal mucosa or alterations of the flagellin epitopes during the encapsulation process. However, the former is more likely than the latter, as Western blotting of flagellin released from the microparticles demonstrated that the released flagellin retained at least a proportion of its B-cell epitopes.

Western blotting demonstrated that the majority of antibody responses induced following vaccination were directed against H7 flagellin. This suggests that the slight protective responses observed in this study were primarily due to immune responses against the flagellin protein. However, we cannot rule out the possibility that other minor contaminants within the vaccine preparation, such as LPS, may have contributed to the altered bacterial shedding observed in i.m. immunized calves. Never-
thelass, by purifying flagellin from a stx mutant LEE4 deletion mutant strain of *E. coli* O157:H7, we could at least be confident that our vaccines were not contaminated with proteins encoded by the LEE4 operon, known to be required for epithelial colonization (32), or Shiga toxin.

Although different strains of *E. coli* O157:H7 were used for in vitro binding assays and oral challenges, which may differ slightly in their epithelial binding dynamics, both strains shared identical H7 flagellin sequences. Therefore, the observation that both Iga and IgG isotypes were effective at inhibiting bacterial binding in vitro in an H7-specific manner suggests that these anti-H7 antibodies may have been similarly effective at inhibiting bacterial epithelial binding in vivo. As both IgA and IgG isotypes are key immunoglobulins present in bovine intestinal secretions (6, 33), this raises two possible explanations for the altered bacterial shedding observed following i.m. immunization but not rectal immunization with H7. First, anti-H7 antibodies may be required throughout the whole intestinal tract and not just at the rectum to have an effect on subsequent *E. coli* O157:H7 colonization in vivo. This appears likely, as although *E. coli* O157:H7 preferentially colonizes at the terminal rectal mucosa, the bacterium also colonizes at other intestinal sites, including the foregut and cecum, and colon (4, 8, 31). Secondly, as combined levels of H7-specific IgG and Iga antibodies were higher at the rectal mucosa following i.m. immunization than following rectal H7 immunization, it may be that the overall quantity of anti-H7 antibodies at the terminal rectum is crucial for altering bacterial colonization in vivo. In either case, the effect of i.m. immunization with H7 appeared to be restricted to either a reduction in initial bacterial uptake or a delay in peak colonization but had no effect on the ability of the bacteria to reach high levels in colonized calves.

It was shown in this study that anti-H7 antibodies resulted in around a 50% reduction, but not complete elimination, of bacterial epithelial binding in vitro. This suggests that *E. coli* O157:H7 possesses additional mechanisms for intestinal epithelial attachment beyond H7-flagellin-mediated processes. Indeed, a recent study in cattle has demonstrated that knocking out the gene encoding H7 flagellin (flfC) had no significant effect on subsequent colonization of cattle with *E. coli* O157, indicating that intestinal epithelial binding in vivo can occur independently of H7 (11). Therefore, the results from this study indicate that although H7 flagellin may be a useful component in a systemic vaccine against *E. coli* O157:H7 in cattle, additional bacterial antigens would need to be included to significantly reduce total bacterial fecal shedding.

An interesting finding in this study is that an H7-specific mucosal IgA response was induced in both nasal secretions and rectal-swab samples following i.m. immunization with H7. This finding is unusual, as systemic routes of immunization generally result in poor mucosal IgA levels (23, 24). However, confidence in the result can be gained by the following observations: first, previous analyses of the mucosal sampling techniques employed in this study have shown that the majority of IgA present in the mucosal samples is locally, i.e., mucosally derived (29). Secondly, analysis of gel filtration fractions of nasal secretions from i.m. immunized calves indicate that the anti-bovine IgA antibodies employed in the H7 ELISA do not appear to cross-react with bovine IgG to any great extent.

The underlying mechanisms of mucosal response generation following systemic H7 immunization are unclear. It may be that the calves in this study were mucosally primed to either H7 flagellin or other *E. coli* flagellins, which share highly conserved regions at the N and C termini (42), prior to immunization, as it has been shown that prior mucosal priming is required to generate an intestinal antigen-specific IgA response to systemic immunization with either trinitrophenyl-keyhole limpet hemocyanin or inactivated polio vaccine in mice and humans, respectively (18, 21). A second possibility is that the mucosal IgA response induced by systemically administered H7 represents an inherent capability of H7 to direct immune responses toward the mucosal surfaces.

Finally, further intriguing findings in this study relate to the dynamics of the antibody response generated by i.m. immunization of H7. First, peak IgA levels occurred after the second immunization in both serum and nasal secretions, whereas H7-specific IgG continued to rise after the third immunization. One possible explanation is that flagellin administered on the third immunization was absent or blocked by preexisting antibodies and therefore was not available to stimulate further antibody production. The subsequent fall in H7-specific IgA, but not IgG, could be explained by differences in their respective half-lives: serum half-lives of IgA and IgG in calves have been estimated to be approximately 2.5 days for IgA and 16 days for IgG (5). Therefore, if the third immunization failed to induce any further antibody production, H7-specific IgA would fall whereas IgG levels would be maintained for a longer period.

Secondly, whereas oral bacterial challenge with *E. coli* O157:H7 resulted in an increase in H7-specific IgA antibodies in mucosal and serum samples from unvaccinated and mucosally immunized calves, no such increase in anti-H7 IgA occurred in the i.m. immunized group. However, as a significant positive correlation existed between levels of anti-H7 and anti-O157 LPS IgA in postchallenge rectal-swab samples from both systemically immunized and nonvaccinated calves, it is likely that the lack of an obvious increase in anti-H7 IgA in the i.m. immunized group was, at the rectum at least, due to lower levels of bacterial colonization. The lack of a correlation between levels of H7 and LPS IgA in postchallenge serum and nasal secretions from i.m. immunized calves could be explained by the existence of high levels of vaccine-induced anti-H7 IgA antibodies in these samples prior to oral challenge.

In conclusion, we have shown that systemic, but not rectal, immunization of cattle with H7 flagellin resulted in reduced colonization rates and delayed peak shedding of *E. coli* O157:H7 following experimental challenge but did not affect total bacterial fecal shedding. This effect was associated with a widespread systemic and mucosal antibody response. Furthermore, both anti-IgA and IgG antibodies were shown to inhibit binding of *E. coli* O157:H7 to the intestinal epithelium in vitro. These results indicate that H7 flagellin may be a useful component of a systemic vaccination against *E. coli* O157:H7 in cattle.

**ACKNOWLEDGMENTS**

This work was supported by DEFRA-Novartis LINK project number LK0666. J.F.H. and D.G.E.S. receive funding from SEERAD.


