WR-192-92

AD-A273 607

REPORT DOCUMENTATION PAGE

Form Approved
OMB No 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED

4. TITLE AND SUBTITLE
E. coli Strain RDEC-1 ←
Pili in Microspheres Protect rabbits from diarrhoea

5. FUNDING NUMBERS

6. AUTHOR(S)
C.E. McQueen, E.C. Boedeker and R. Reid

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Walter Reed Army Institute of Research
Washington D.C. 20307 - [redacted] 5100

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
US Army Medical Research + Development Command
Ft. Detrick, Frederick Md 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Available for public release;
Distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 words)

We tested whether pilus proteins of rabbit diarrhoeagenic Escherichia coli *(RDEC-1), incorporated into biodegradable microspheres, could function as safe and effective oral immunogens in the rabbit diarrhoea model. The RDEC-1 adhesin, AF/R1, incorporated into poly*(D,L-*lactide*-co-*glycolide*) *microspheres, was administered intraduodenally. Vaccinated and unvaccinated rabbits were challenged with RDEC-1 and killed 1 week later. Vaccination with AF/R1 in microspheres did not cause diarrhoea or weight loss. After challenge, rabbits given AF/R1 in microspheres, in contrast to unvaccinated animals, remained in good health. RDEC-1 attachment to caecal epithelium of vaccinated rabbits was reduced* ($p = 0.02$), *whereas numbers of RDEC-1 in intestinal fluids were little affected. Also, in vaccinated animals, biliary anti-AF/R1 IgA levels were increased, and AF/R1-induced blast-cell transformation was vigorous in spleen cell cultures. We conclude that vaccination with AF/R1 in microspheres was safe and protected rabbits against RDEC-1 disease, probably by interfering with adherence of the bacteria to the intestinal mucosa. The interference might have been due to the presence of specific antibodies secreted in bile.*

14. SUBJECT TERMS

15. NUMBER OF PAGES

Keywords: Enteric vaccine; mucosal immunity; biliary IgA; mucosal attachment; enteroadherent *Escherichia coli*

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRAC

Encl 3

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)

Pili in microspheres protect rabbits from diarrhoea induced by *E. coli* strain RDEC-1*

C.E. McQueen[†§], E.C. Boedeker[†], R. Reid[†], D. Jarboe[†], M. Wolf[†], M. Le[‡] and W.R. Brown[‡]

We tested whether pilus proteins of rabbit diarrhoeagenic Escherichia coli *(RDEC-1), incorporated into biodegradable microspheres, could function as safe and effective oral immunogens in the rabbit diarrhoea model. The RDEC-1 adhesin, AF/R1, incorporated into poly(D,L-lactide-co-glycolide) microspheres, was administered intraduodenally. Vaccinated and unvaccinated rabbits were challenged with RDEC-1 and killed 1 week later. Vaccination with AF/R1 in microspheres did not cause diarrhoea or weight loss. After challenge, rabbits given AF/R1 in microspheres, in contrast to unvaccinated animals, remained in good health. RDEC-1 attachment to caecal epithelium of vaccinated rabbits was reduced* ($p = 0.02$), *whereas numbers of RDEC-1 in intestinal fluids were little affected. Also, in vaccinated animals, biliary anti-AF/R1 IgA levels were increased, and AF/R1-induced blast-cell transformation was vigorous in spleen cell cultures. We conclude that vaccination with AF/R1 in microspheres was safe and protected rabbits against RDEC-1 disease, probably by interfering with adherence of the bacteria to the intestinal mucosa. The interference might have been due to the presence of specific antibodies secreted in bile.*

Keywords: Enteric vaccine; mucosal immunity; biliary IgA; mucosal attachment; enteroadherent *Escherichia coli*

INTRODUCTION

RDEC-1 is a mucosally adherent strain of *E. coli* that causes a diarrhoeal infection in rabbits and provides a model for the study of adherence factor-based immunity[1]. RDEC-1 attach to mucosal epithelial cells by adhesins in a multi-step process that has early and late phases[2]. The AF/R1 pilus adhesin is a well described virulence factor for RDEC-1 that functions during the early phase of attachment[2].

Pilus adhesins are logical candidates for inclusion in vaccines designed to protect against adherent enteric pathogens since blocking of the adhesin–receptor interaction by antibodies may interfere with attachment of the organisms to the mucosa. However, purified pili administered orally have not proven successful as oral vaccines. This failure may be the result of degradation of the pilus antigens during passage through the gastrointestinal tract[3] or inadequate uptake of the antigens by gut-associated lymphoid tissue.

A novel approach for enhancing the mucosal immunogenicity of antigens relevant to vaccine development is incorporation of the antigens into microspheres composed of biodegradable, biocompatible lactide/glycolide polymers[4–8]. Delivery of antigens via microspheres may enhance the mucosal response to the antigens by a number of mechanisms, including protecting them from digestion during passage through the gastrointestinal tract and by targeting them to lymphoid cells in Peyer's patches[4–6].

In this work, we explored the potential of AF/R1 pili administered enterically in microspheres to safely protect rabbits against challenge with RDEC-1.

MATERIALS AND METHODS

Bacteria

Nalidixic acid-resistant RDEC-1, isolated from laboratory rabbits that spontaneously developed diarrhoea[9,10], were grown overnight under conditions that promote the expression of AF/R1 pili (in Penassay broth)[11]. The bacteria were pelleted by centrifugation, washed twice and resuspended in PBS to give a final count of 10^{8-9} viable RDEC-1 ml^{-1}.

Preparation of RDEC-1 pili

AF/R1 pili were prepared by a described method[12]. RDEC-1 grown in static Penassay broth overnight were harvested by centrifugation, resuspended in Tris buffer,

*Parts of these data were presented during Digestive Disease Week at the 92nd Annual Meeting of the American Gastroenterological Association, 19–22 May 1991, New Orleans, LA, and were published in *Gastroenterology* 1991, **100**(5), A599.

[†]The Department of Gastroenterology, Walter Reed Army Institute of Research (Bldg 40), Washington, DC 20307, USA. [‡]The Department of Medicine, Veterans Administration Medical Center and University of Colorado School of Medicine, Denver, CO 80220, USA. [§]To whom correspondence should be addressed.

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and homogenized with a Sorval Omnimixer (Dupont Instruments, Wilmington, DE) to shear pili. Intact bacteria were removed by centrifugation, and pili were precipitated from the supernatant by the stepwise addition of ammonium sulphate and centrifugation. The pelleted pili were resuspended in distilled water, dialysed overnight, and lyophilized. Eight separate preparations of 10–15 mg protein, each documented to contain >90% AF/R1 pilus subunit, were pooled for incorporation into microspheres. Purity of each preparation was documented by scanning laser densitometry after electrophoresis in SDS–polyacrylamide gels and staining with Coomassie blue. Ninety-two per cent of the protein in the final pool of 100 mg protein migrated as a single band at 19 kDa, consistent with the known molecular weight of AF/R1 pilus subunits[12].

Preparation of AF/R1 in microspheres for immunization

DL-Lactide and coglycolide microspheres incorporating purified AF/R1 pili were prepared by the Southern Research Corporation, using an emulsion-based process which is a modification of a solvent-extraction process described in detail elsewhere[13]. The size and pilus protein content of the microspheres were as specified by the United States Army Medical Research and Development Command (USAMRDC): 50–100 mg to provide 100 μg doses of incorporated protein in 5–10 μm capsules. Prior to the final encapsulation, aliquots of the AF/R1 preparation were shown to retain antigenicity (double sandwich ELISA) and characteristic electrophoretic mobility of the pilin subunit upon treatment with each of the solvents. The final product consisted of spherical particles (AF/R1-MS), ranging from 1 to 12 μm and containing 0.62% AF/R1 pilus protein by weight. The AF/R1-MS were sterilized at room temperature by gamma irradiation (0.3 megarads) and stored at 4°C in a desiccator. The immunogenicity of the AF/R1 pilus preparation was retained after incorporation into microspheres, as demonstrated by the induction of a secondary serum IgG antibody response to the pilin subunit following intramuscular (i.m.) injection of sonicated (Bronson Ultrasonic Cleaner 3200, Danbury, CT) AF/R1-MS in PBS into rabbits previously infected with RDEC-1.

Rabbits

New Zealand white rabbits, 1 kg in weight, obtained from closed colonies maintained at the National Institutes of Health, Bethesda, MD, were observed for 1 week at Walter Reed quarantine facilities and selected for study if they (i) did not have measurable serum antibodies at a 1:2 dilution to RDEC-1 antigens (by ELISA), (ii) were not colonized by *E. coli* (as determined by cultures of rectal swabs), and (iii) did not have diarrhoea (as indicated by 'wet tail', perirectal soiling, or unformed stool in the cage bottom).

Vaccination and challenge of rabbits

Rabbits were allocated to one of three groups: vaccinated and challenged with RDEC-1 ($n=6$); unvaccinated and challenged with RDEC-1 ($n=9$); vaccinated and not challenged ($n=2$). For the first inoculation, 34 mg of AF/R1 pili in microspheres (AF/R1-MS) containing 200 μg of AF/R1 were injected through an Olympus BF type P10 bronchoscope into the duodenum of rabbits that had been fasted overnight and sedated with an intramuscular injection of Ketamine HCl (50 mg i.m.) (Ketaset, Fort Dodge Laboratories, Fort Dodge, IA) and xylazine (10 mg i.m.) (Rompun, Mobay Corporation, Shawnee, KS). The endoscope was advanced under direct vision into the stomach, which was insufflated with a 50 ml bolus of room air via a catheter passed through the biopsy channel. The catheter was advanced through the pylorus 3–4 cm into the duodenum, and the microsphere suspension in 1 ml of PBS was injected, followed by a 9 ml flush of PBS. Rabbits in the vaccinated-challenged group were given intraduodenal booster injections of 17 mg AF/R1-MS containing 100 μg of AF/R1 at weekly intervals for 3 weeks. One week after the last booster dose, 10^{8-9} RDEC-1 in 1 ml PBS and 9 ml of 10% $NaHCO_3$ solution (to neutralize gastric acid) were given by orogastric tube. The animals were killed 1 week later. Rabbits in the unvaccinated-challenged group were given 10^{8-9} RDEC-1 and killed after 1 week. The vaccinated-unchallenged group was vaccinated as in group 1 and killed 1 week after the last booster dose. All animals were monitored daily for signs of illness (weight loss, perineal soiling, unformed pellets or scruffy appearance).

Collection of intestinal fluids and tissues

Animals were sedated with ketamine and xylazine and immediately overdosed with sodium pentobarbital (V Pento, A.J. Buck, Cockeysville, MD). The abdomen was opened, and bile was obtained from the gall bladder via a 27-gauge needle. The bile was immediately chilled on ice and stored at −70°C. Samples were taken from isolated segments of duodenum, jejunum, ileum and proximal and distal colon, as well as the caecum, as described[14].

Cultures

The intestinal fluid samples were vortexed, and ten tenfold serial dilutions were made in PBS; 0.1 ml aliquots were spread on nalidixic acid-treated agar plates, and colonies were counted after 24 h incubation.

Determination of mucosal anti-RDEC-1-antibody-containing cells and adherent RDEC-1

Anti-RDEC-1-antibody-containing cells were identified as we have described[14]. Cryostat sections of intestine that had been fixed in periodate-lysine-paraformaldehyde were reacted with RDEC-1 pilus preparation[12] for 2 h, washed with PBS, and reacted with horseradish peroxidase-labelled goat $F(ab')_2$ anti-RDEC-1 antibodies. For control sections, PBS was substituted for RDEC-1 pili, and peroxidase-labelled $F(ab')_2$ fragments prepared from non-immune goat gamma globulin were substituted for the specific antibodies. Positive-reacting cells were enumerated by counting through a light microscope grid.

For identification of enteroadherent RDEC-1, similarly prepared sections were reacted as just described, except that incubation with the RDEC-1 pilus preparation was omitted. Adherence was semi-quantified by visual examination according to our previously described scoring system[2]: +1, focal attachment to single cells; +2, diffuse attachment over the apical surface of several cells; and +3, confluent attachment.

Measurement of anti-pilus IgA in bile

IgA anti-pilus antibodies in bile were measured by ELISA, as described[14], in which the reference standard was bile collected from rabbits during week 2 of an

RDEC-1 infection, and bile collected from two animals that were given 10 ml of 10% $NaHCO_3$ by gastric tube and killed 1 week later was used as a negative control. Antibody concentration was expressed in ELISA units.

Anti-AF/R1-stimulated blast-cell transformation

A mononuclear suspension of cells from the Peyer's patch nearest to the ileocaecal valve was prepared by gently passing the cells through a 19-gauge needle and syringe. A mononuclear suspension of spleen cells was prepared by mincing the spleen with a razor blade and gently passing the cells through a 100-mesh wire gauze. The splenic and Peyer's patch cell suspensions were washed in supplemented Dulbecco's modified Eagle medium (cDMEM) (Gibco Laboratories, Grand Island, NY), containing penicillin (100 units ml^{-1}), streptomycin (100 $\mu g\,ml^{-1}$), L-glutamine (2 mM), and HEPES Buffer (10 mM) (all obtained from Gibco Laboratories), as well as MEM non-essential amino acid solution (0.1 mM), MEM (50X) amino acids (2%), sodium bicarbonate (0.06%), and 5×10^{-5} M 2-mercaptoethanol (all obtained from Sigma Chemical Company, St Louis, MO). Erythrocytes remaining in the spleen-cell preparation were lysed with filter-sterilized ammonium chloride-containing buffer. Mononuclear cells were suspended at a concentration of 5×10^6 cells ml^{-1} in supplemented Dulbecco's modified Eagle medium (cDMEM), and autologous serum was added to a final concentration of 0.5%. Amounts of 100 ml of cell suspensions and purified AF/R1 pilus preparation, in concentrations ranging from 150 ng to 15 $\mu g\,ml^{-1}$, were pipetted into 96-well flat-bottomed microtitre culture plates (CoStar, Cambridge, MA) and incubated at 37°C in 5% CO_2 for 4 days. Cultures performed in quadruplicates were pulsed with 1 μCi [^{3}H]thymidine (25 Ci $mmol^{-1}$, Amersham, Arlington Heights, IL) and harvested for scintillation counting 6 h later. Data are expressed as a stimulation index, calculated by dividing the mean scintillation counts of cultures in the presence of antigen by the mean scintillation counts of cultures in the absence of antigen (media control). A positive response to AF/R1 stimulation was defined as a stimulation index >2.

Statistical analysis

A two-tailed t test for unpaired data or the Wilcoxon Rank Sum test was used to compare the means of study groups.

RESULTS

Assessment of illness

Like unvaccinated animals, none of the six animals vaccinated with AF/R1-MS became ill during the weeks between inoculation with AF/R1-MS and challenge with RDEC-1, and were gaining weight (*Figure 1*). After RDEC-1 challenge, only one vaccinated animal lost weight after challenge (percentage change in body weight: −2), and only one shed soft (but formed) pellets for 1 day only (day 2). In contrast, all unvaccinated animals lost weight and shed soft pellets by day 4 after challenge; one animal developed unformed, mucoid stools. In contrast, unvaccinated animals lost an average of 13% of their body weight during the week after challenge. The difference in mean percentage change of body weight between vaccinated and unvaccinated groups of animals was significant (*Figure 1*).

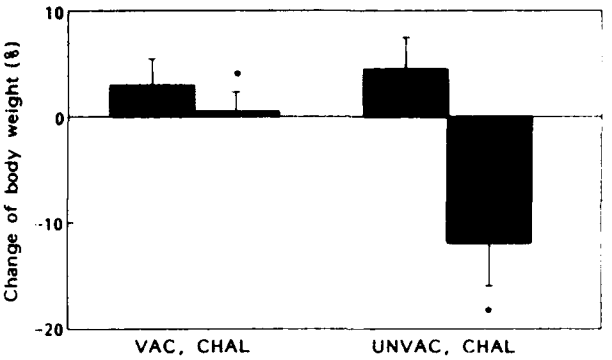

Figure 1 Mean percentage changes in body weight of rabbits for the week preceding (■) and the week following (■) challenge with RDEC-1. VAC, CHAL: vaccinated with AF/R1-MS, challenged with RDEC-1; UNVAC, CHAL: unvaccinated, challenged with RDEC-1. Values are means ± s.d. *, $p < 0.004$, two-tailed t test for unpaired data

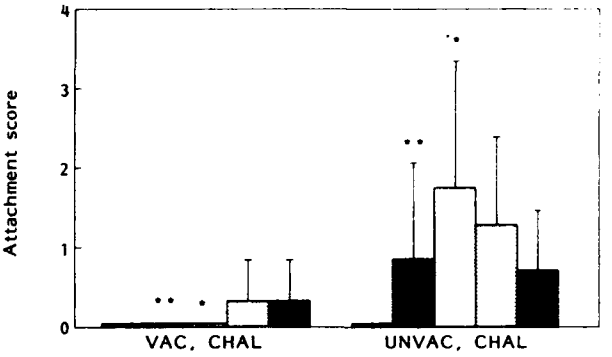

Figure 2 Mean scores for attachment of RDEC-1 to intestinal epithelial cells 1 week after challenge with RDEC-1. VAC, CHAL: vaccinated with AF/R1-MS, challenged with RDEC-1; UNVAC, CHAL: unvaccinated, challenged with RDEC-1. RDEC-1 attachment to epithelial cells in histological sections was graded as described in the methods section. ■, Jejunum; ■, ileum; □, caecum; ▤, proximal colon; ■, distal colon. Scores are expressed as the mean ± s.d. *, $p < 0.02$, **, $p < 0.1$ (two-tailed t test for unpaired data). To enhance the visual clarity of the graph, intestinal segments containing no attached organisms were given an attachment score of 0.1

Attachment of RDEC-1 to mucosal epithelial cells

RDEC-1 were often seen attached to ileal or caecal epithelial cells in unvaccinated animals killed 1 week after challenge, whereas RDEC-1 were rarely seen attached to epithelial cells in these segments in vaccinated animals (*Figure 2*). The difference in attachment scores in the caecum of vaccinated and unvaccinated animals was significant ($p < 0.02$). Greater numbers of attached organisms were also present in the ileum and proximal colon of unvaccinated compared with vaccinated animals, but these differences were not statistically significant. RDEC-1 were not seen attached to jejunal epithelial cells in either vaccinated or unvaccinated animals.

Colonization

Luminal colonization with RDEC-1 after challenge was not significantly different between the vaccinated and unvaccinated groups, except for a two log lower count in the jejunum of vaccinated animals (*Figure 3*).

Anti-AF/R1 antibody in bile

Anti-AF/R1 antibody concentrations were greater than one standard deviation (s.d.) over negative control values in both of the AF/R1-MS vaccinated, unchallenged

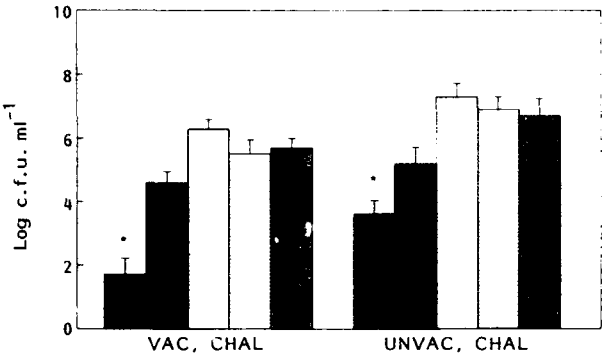

Figure 3 RDEC-1 colonization of luminal contents in AF/R1 vaccinated and unvaccinated animals 1 week after RDEC-1 challenge, expressed as mean colony-forming units (c.f.u.) ($\log_{10}$) ± s.e. VAC, CHAL: vaccinated with AF/R1-MS, challenged with RDEC-1; UNVAC, CHAL: unvaccinated, challenged with RDEC-1. Symbols as in *Figure 2*. *, $p = 0.03$, Wilcoxon Rank Sum Test

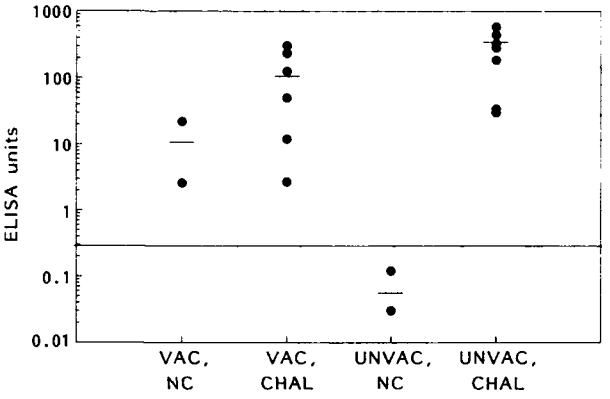

Figure 4 Anti-AF/R1 pilus IgA antibodies in bile measured by ELISA. VAC, NC: vaccinated with AF/R1-MS, not challenged with RDEC-1; VAC, CHAL: vaccinated with AF/R1-MS, challenged with RDEC-1; UNVAC, NC: unvaccinated, not challenged; UNVAC, CHAL: unvaccinated, challenged with RDEC-1. The horizontal line indicates 1 s.d. above the mean value for the unvaccinated, not challenged (negative control) rabbits. Each point represents the anti-AF/R1 antibody level for one animal

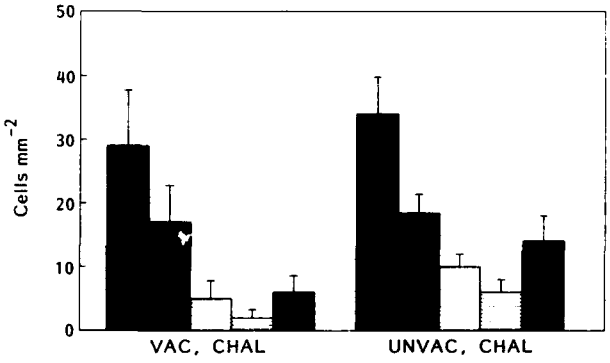

Figure 5 Anti-AF/R1 antibody-containing cells in histological sections of intestine. VAC, CHAL: vaccinated with AF/R1-MS, challenged with RDEC-1; UNVAC, CHAL: unvaccinated, challenged with RDEC-1. Symbols as in *Figure 2*. Scores are expressed as the mean cells mm^{-2} ± s.d. of lamina propria

animals 1 week after vaccination (*Figure 4*). In both the AF/R1-MS vaccinated and unvaccinated groups, a vigorous antibody response, to about the same level, occurred after challenge with RDEC-1.

Anti-AF/R1 antibody-containing cells in the lamina propria

Before challenge with RDEC-1, anti-AF/R1 antibody-containing cells were not seen in immunoperoxidase-stained histological sections of the intestinal lamina propria in animals either vaccinated with AF/R1-MS or not vaccinated. After challenge, specific antibody-forming cells were present in both groups, without significant difference between the groups in the numbers of cells in any region of the gut (*Figure 5*).

AF/R1 pilus antigen-stimulated blast-cell transformation

A vigorous blast-cell transformation in response to AF/R1 pilus protein occurred in cultures of mononuclear spleen cells from both of the animals that were vaccinated with AF/R1-MS but not challenged with RDEC-1 (stimulation index: 30 and 4). In similar cultures of Peyer's patch cells, a blast-cell transformation occurred in one of the two animals (stimulation index: 3); this animal also had the higher anti-AF/R1 antibody level in bile.

DISCUSSION

Optimal mucosal immunization by vaccines appears to require mucosal administration of antigen[15,16]. However, protein-based vaccines are generally weak mucosal immunogens when the antigen is administered in purified, soluble form, perhaps because of digestion of the antigen within the bowel lumen or poor uptake of it by gut-associated lymphoid tissue. Previously, we found that treating purified CFA/II, an adhesin complex isolated from enterotoxigenic *E. coli* with 0.1 M HCl and pepsin (conditions intended to mimic those in the stomach) caused rapid degradation of the CFA/II protein to small peptides and loss of antigenicity[17]. Conceivably, AF/R1 pilus protein administered orally to rabbits undergoes at least as extensive degradation *in vivo*. Nevertheless, AF/R1 pili are potential vaccine candidates, since a mucosal antibody response to AF/R1 was stimulated in rabbit Thiry-Vella loops by inoculation of the loops with the pili[18].

In living tissue, filaments composed of lactide–coglycolide copolymers are non-antigenic, non-pyrogenic, non-toxic[19], and degrade over time at rates largely determined by the molar ratios of the lactide to glycolide[19]. The inertness and orderly degradation of lactide–coglycolide copolymers in tissue has led to their use since the 1970s in resorbable surgical sutures. These properties have also prompted the testing of lactide–coglycolide copolymers for a wide variety of additional uses[19], including as carriers for drugs, hormones and antigens.

Enhancement of the mucosal immune response by incorporating antigens into microspheres composed of a lactide–glycolide copolymer has been reported[4–8]. Several mechanisms have been hypothesized to account for the immune enhancement: protection of antigens from proteolytic digestion, more efficient uptake of antigens by gut-associated lymphoid tissue and antigen-presenting cells, and persistence of critical-sized microspheres in the Peyer's patch, with sustained release of antigen.

Eldridge *et al.*[4] reported markedly increased anti-enterotoxin IgA titres in gut fluids of mice orogastrically given microspheres incorporating staphylococcal enterotoxin B compared with titres in mice given soluble toxin. Microsphere particles $< 10\,\mu m$ in diameter entered the

Peyer's patches, and microspheres > 5 μm persisted there, thus perhaps providing prolonged antigenic stimulation. Intraperitoneal priming with orogastric boosting was nearly as effective as tertiary oral immunization for stimulating mucosal anti-toxin antibodies[6]. In another study, mice orally given microspheres incorporating influenza developed a partially protective, antigen-specific antibody response in secretions[7]. It was reported in an earlier work[8] that cultures of Peyer's patch cells prepared from rabbits immunized intraduodenally with AF/R1-MS had increased AF/R1-induced proliferation compared with cultures from animals immunized with AF/R1. Also, cultures of Peyer's patch cells from animals immunized with AF/R1-MS, but not AF/R1, proliferated in response to synthetic peptides representing probable B- and T-cell epitopes of the AF/R1 pilus (Jarboe, unpublished results). However, an enhanced anti-AF/R1 antibody response was not observed in intestinal fluids after immunization with microspheres containing incorporated AF/R1[20], perhaps because insufficient amounts of pili were given or the time period (3 weeks) after inoculation was too short.

In the present study, rabbits were protected from RDEC-1 diarrhoeal disease for at least 1 week after four intraduodenal doses of AF/R1-pilus antigen incorporated into microspheres. Evidence of the protection was the absence of weight loss and poorly formed stools in the vaccinated animals. A marked decrease in the numbers of RDEC-1 attached to distal small intestinal and caecal mucosa was a feature in the protected animals, even though there were no significant differences in the numbers of RDEC-1 populating the intestinal fluids in those regions of the bowel. Although the numbers of RDEC-1 in the jejunum of vaccinated, challenged animals was modestly (2 logs) lower than in the unvaccinated animals, the biological relevance of the difference is arguable. It was previously demonstrated in this laboratory that piliated (but not non-piliated) RDEC-1 coaggregate *in vitro* with rabbit glycoprotein preparations from luminal mucus[21]. The lack of correlation between decreased adherence and luminal colonization is consistent with the interpretation that luminal glycoproteins in the mucous layer serve as a site for bacterial colonization before bacteria adhere to the mucosa. That protection of the rabbits correlated well with decreased adherence of RDEC-1 is consistent with the opinion that attachment of the bacteria to mucosal epithelial cells, rather than simply colonization of the lumen, accounts for the diarrhoeal illness they produce[22]. In this regard, Tacket *et al.*[23] described clinical protection from enterotoxigenic *E. coli*-induced diarrhoea through the ingestion of milk immunoglobulin concentrate, despite colonic colonization with the bacteria.

Our data do not make entirely clear the reason for the decrease in mucosal attachment of RDEC-1 in vaccinated rabbits, although anti-pilus antibodies in bile are implicated. In bile of the two animals vaccinated with microspheres, specific anti-AF/R1 pilus antibodies were present at levels 1–2 logs above the upper limit of values in negative control animals; even though the antibody levels in the vaccinated animals were only about 1/10 to 1/30 the levels present in animals challenged with RDEC-1, perhaps sufficient antibodies were still present to bind to RDEC-1 and interfere with their attachment to the mucosa. Whether the biliary antibodies also accounted for the fewer RDEC-1 in the jejunal fluids of challenged animals is an interesting consideration. In our previous study[14], secretion of anti-RDEC-1 antibodies into bile accounted for the vast majority of specific antibodies in the intestinal lumen. Thus, the biological significance of the biliary antibodies in this model may be great.

The source of the biliary anti-AF/R1 pilus antibodies was not identified. In the rabbit, a large proportion of polymeric IgA antibodies synthesized in the intestinal mucosa enter the mesenteric lymph and are cleared by the liver into bile[24]. Even though we could not detect anti-AF/R1 plasma cells in the intestinal lamina propria of rabbits vaccinated with microspheres (but not challenged with RDEC-1), perhaps there were sufficient cells present to account for the biliary antibodies. Alternatively, specific antibody-forming cells may have been present in the liver, since accumulation of antibody-forming cells in the livers of animals immunized with other antigens has been demonstrated[25,26].

We demonstrated a strong proliferative response to the AF/R1 antigen in cultures of mononuclear cells from the spleens of both animal vaccinated with AF/R1-MS (but not challenged) and in cells from the Peyer's patch of one of the animals. Although the majority of the responding lymphocytes were probably T cells, additional studies would be required to conclusively establish their identity. Sensitization of T cells of the helper phenotype probably precedes the development of a mucosal antibody response to the AF/R1 antigen, and proliferation of these cells in response to AF/R1 antigen in culture confirms that they were sensitized to the AF/R1 antigen. Even though we did not see anti-AF/R1-containing cells in the lamina propria of vaccinated but unchallenged animals, it is possible that 4 weeks of immunization is insufficient time for accumulation of anti-AF/R1-containing cells to a level that can be detected by immunohistochemical methods, or that the antibody-forming cells localized mainly to the liver.

In this initial attempt to immunize intact rabbits by the enteric administration of AF/R1 pili in lactide/glycolide polymer microspheres, immunization was safe and provided at least short-term protection. Further investigation of this promising method of vaccine delivery is indicated. We gave AF/R1-MS by the intraduodenal route primarily to assure their delivery into the small intestine and to avoid degradation by gastric juice. In future studies, we will attempt to determine the duration of protection induced. In immunization against enteric pathogens, it may be desirable to prevent luminal colonization as well as protection. In the RDEC-1 model, perhaps this level of immunization could be achieved by enhancing the immunogenicity of the microspheres by incorporating into them other surface antigens, such as lipopolysaccharide[27] or other critical gene products[28], together with AF/R1.

ACKNOWLEDGEMENTS

The authors acknowledge the expert technical support provided by Omar Bilal, Eloyse Fleming, Joe Gomez, James Kodak and Evelyn Lee, and Craig Morrissette for assistance with the statistics. Funding for this study was provided in part by a collaborative grant from the United States Department of Defense and Department of Veterans Affairs. © US Government.

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