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Intranasal Vaccination in Mice with an Attenuated *Salmonella enterica* Serovar 908htr A Expressing Cp15 of *Cryptosporidium*: Impact of Malnutrition with Preservation of Cytokine Secretion

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Abstract

Cryptosporidium is a protozoan parasite associated with acute and persistent diarrhea that, even in asymptomatic persons, can impair normal growth and potentially cognitive and physical development in young children. The recent availability of the complete gene sequence for *C. hominis* antigen Cp15 allows examination of innovative vaccine regimens involving intra-nasal antigen priming with live bacterial vectors applicable to human populations. We used a recently described weaned mouse model of cryptosporidiosis, where nourished and malnourished vaccinated mice receive the Cp15 antigen recombinant with cytolysin A on a *Salmonella* serovar Typhi CVD 908-htrA vector, followed by parenteral exposure to antigen with adjuvant. After challenge with *Cryptosporidium* oocysts via gavage, parameters of infection and disease (stool shedding of parasites, growth rates) were quantified, and serum/lymphoid tissue harvested to elucidate the Cp15-specific adaptive immune response. In vaccinated nourished mice, the regimen was highly immunogenic, with strong antigen-specific IL-6 and IFN- γ secretion and robust Cp15-specific immunoglobulin titers. In vaccinated malnourished mice, secretion of cytokines, particularly IFN- γ , and antigen-specific humoral immunity were generally undiminished despite protein deprivation and stunted growth. In contrast, after natural (oral) challenge with an identical inoculum of *Cryptosporidium* oocysts, cytokine and humoral responses to Cp15 were less than one-fourth those in vaccinated mice. Nevertheless, vaccination resulted in only transient reduction in stool shedding of parasites and was not otherwise protective against disease. Overall, immunogenicity for a *C. hominis* antigen was documented in mice, even in the setting of prolonged malnutrition, using an innovative vaccine regimen involving intra-nasal antigen priming

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with a live enteric bacterial vector, that has potential applicability to vulnerable human populations irrespective of nutritional status.

Keywords

C. parvum; vaccine; Cp15; intra-nasal priming; malnutrition

1. Introduction

Cryptosporidiosis is the cause of significant morbidity and mortality worldwide [1,2]. The leading human species, *C. hominis* and *C. parvum*, infect the microvillus border of the gastrointestinal epithelium, but the latter infects a wider range of vertebrate hosts including humans, causing acute, persistent and/or chronic diarrhea [3]. These pathogens are spread through ingestion of contaminated water and food, exposure to infected animals, as well as by fecal-oral contact [4].

With regard to adaptive immunity, infection with *Cryptosporidium* elicits a humoral immune response in immune-competent persons and in patients with AIDS, although that response is not associated with clearance of infection [5]. Cell-mediated immunity is regarded as essential to clearance of the parasite [6], where IFN- γ , secreted primarily by the Th1 subset of CD4⁺ lymphocytes, is particularly crucial for eradication of *Cryptosporidium* [7]. Thus, mice can be rendered susceptible to experimental cryptosporidiosis if they are pre-treated with antibody to IFN- γ or are immune-suppressed with dexamethasone. Resistance is re-established in these mice by administration of high doses of IFN- γ [8,9].

Detailed knowledge of the immune response to this parasite is important for the development of a successful vaccination regimen to protect humans against cryptosporidiosis. However, these endeavors face two major challenges. First, it is essential to develop an immunization regimen that elicits a potent immune response, optimally cell-mediated, that is also induced at least in part in the intestinal mucosa and that is appropriate for administration to humans with an acceptable side effect profile. A *Salmonella* live vector system was chosen for the studies reported herein, using two immunization protocols (Regimens I and II, Table 1). These protocols combine intra-nasal delivery of a *Salmonella* enteric serovar 908 htrA ClyA vector secreting a model antigen, followed by parenteral administration of the recombinant protein in one of several adjuvants. Second, a parasite-specific antigen, e.g., a protein component of the organism that is expressed early upon entering the intestine and that is required for its invasion of epithelium must be identified, and that also induces a host-protective response.

For malnourished children in developing countries, in whom chronic cryptosporidiosis is common and has been associated with long-term developmental and cognitive deficiencies [10,11], designing a successful immunization regimen is particularly challenging. Previous studies have suggested that global and micro-nutrient malnutrition blunt host-protective immune responses to infectious agents, and could be responsible in part for vaccine failure [12,13]. To address these critical issues of immunogenicity and the impact of malnutrition, we have used a recently described vaccine candidate antigen, designated Cp15 (see below), expressed in and cloned from the previously sequenced *C. hominis* sporozoite [14], as a study antigen, and evaluated its ability to induce an immune response in a new nourished and malnourished mouse model of cryptosporidiosis [15, 16]. Specifically, we sought to determine: 1) the efficacy of a previously developed prime-boost strategy for eliciting a humoral and cell-mediated immune response in the nourished and malnourished host; 2) whether vaccine-induced IFN- γ , crucial for clearing *Cryptosporidial* infection, is diminished

or preserved at the systemic and/or mucosal level in the setting of malnutrition; 3) if there is a difference in the immune response to a *Cryptosporidium*-derived antigen, when the host's exposure is by vaccination compared with that which occurs during 'natural' infection following oral oocyst ingestion; and 4) whether either of two prime-boost vaccine regimens is protective for the host against a *Cryptosporidium* challenge, in terms of lessened growth short-falls and reduced stool shedding of parasites.

2. Materials and Methods (see Supplementary Material)

3. Results

3.1. Weaned mouse model of cryptosporidiosis

As an alternative to prior published efforts (8,9), an experimental model of *Cryptosporidium* infection has been recently reported [15], consisting of weaned C57BL/6 mice which, when malnourished, are vulnerable to weight loss and prolonged stool shedding of parasites after challenge with excysted oocysts. For vaccine studies, this development importantly makes possible *in vivo* studies of new regimens without the need to intentionally deplete crucial elements of the immune response. Therefore, we elected to use this model [15], and a similar one where oral parasite challenge is accomplished with unexcysted oocysts ([16] to explore new regimens (Table I) for eliciting a systemic and mucosal immune response to a candidate vaccine antigen. The three studies reported herein, outlined in Table 1, are basically the same in design, with a modest difference in Regimen II (prime, prime, boost, rather than prime, boost, boost). For all studies, tissues were harvested within 20–40 days of a *C. parvum* challenge for determination of cytokine levels.

3.2. Nutritional status alters the immune response to a *C. parvum*-specific antigen administered with intra-nasal priming using a live bacterial vector

Several regimens for immunization using intra-nasal priming with attenuated *Salmonella* enteric serovar 908htr A have been published [17,18], but only two have studied priming with a *Cryptosporidium*-derived antigen such as Cp15 [19,20]. Unexplored is comparison of a vaccine-generated response with that which occurs when the non-vaccinated host is naturally (orally) infected, as well as what the impact of nutritional status of the host is in altering the immune response to a candidate *C. parvum* vaccinogen. Therefore, nourished and malnourished C57BL/6 mice, with or without administration of Cp15-specific vaccine regimens I or II, as described in Table 1, were euthanized 20–40 days after vaccination was complete, and their serum and lymphoid tissues were harvested for quantification of humoral and cell-mediated immunity to Cp15.

Focusing first on *nourished vaccinated mice*, intra-nasal priming with the attenuated *Salmonella* Serovar CVD 908 htr A vector expressing Cp15 antigen followed by two intra-peritoneal injections with Cp15 protein (Regimen I), elicited solid systemic humoral and cell-mediated immune responses similar to those we previously described [17]. That is, in Study 1 as confirmed in Study 2---both using vaccination Regimen I--- anti-Cp15 antibody titers >1:25,000 and robust cytokine (IL-6, IFN- γ) responses of greater than 1400 pg/ml were observed (Table 2A). The IFN- γ response by splenocytes was more than 2-fold greater than that of the other cytokines tested, while IL-2 secretion was low.

In contrast, natural infection, using *C. parvum* oocyst oral challenge only, elicited a quantitatively different immune response to Cp15 antigen in nourished mice (Table 2 B). In both Studies 1 and 2, regimen I generated a diminished anti-Cp15 humoral and cell-mediated (IL-6, IFN- γ) response to Cp15, with values often less than one-third that observed in nourished vaccinated mice (Table 2A). IL-2 secretion remained low. By these parameters, the prime/boost vaccination regimen is superior to natural infection for

generating in the nourished host a robust humoral and cell-mediated immune response to a *Cryptosporidium*-derived antigen.

The impact of malnutrition on the systemic immune response to a parasite-derived candidate vaccinogen was examined next. In *vaccinated malnourished mice* administered Regimen I, humoral and cytokine responses were generally unimpaired (Table 2C, Study 2, confirmed by Study 1), i.e., their responses were comparable or greater than those of similarly treated nourished mice administered vaccine ($p=0.62$, 0.82 , and 0.59 for IL-2, IL-6, and IFN- γ , respectively, Table 2A versus 2C, Study 2). In particular, secretion of IFN- γ , which is considered crucial for anti-*Cryptosporidial* activity in mice [7], was strong, and, in fact, was at a similar level to that observed when examined in nourished mice (3000 ± 1493 versus 3250 ± 1864 pg/ml; Study 2, Table 2A versus 2C; $p>0.40$). When Regimen II was administered to malnourished mice (Study 3, Table 2C), the results for IL-6 and IFN- γ were similar to those with Regimen I (Study 2, Table 2A; p values for IL-6 and IFN- γ were 0.35 and 0.76 , comparing Study 2, Table 2A with Study 3, Table 2C). These findings suggested that both Regimen I and II could elicit a solid systemic immune responses to vaccine antigen in the malnourished host with efficacy close to that observed in nourished animals.

In contrast, following natural (oral) oocyst challenge, a major difference in cytokine secretion associated with malnutrition was observed, when oocysts excysted (Study 1) or unexcysted (Study 2, Table 2D) were administered. In these mice, only a weak IFN- γ response by splenocytes to Cp15 antigen was observed ($< 1/20^{\text{th}}$ that in vaccinated mice). Similarly, IL-6 secretion ($< 1/3^{\text{rd}}$) and Cp15-specific antibody ($< 1/8^{\text{th}}$) were lower in these mice compared to malnourished vaccinated mice; p values were 1.0 (IL-2), 0.06 (IL-6), and 0.13 (IFN- γ), comparing Study 2 values, Table 2D with those of Study 2, Table 2C. Parallel findings were present when the same mice were compared to those of vaccinated mice of Study 3, RII), ($p>0.14$). These responses were similar to those observed in nourished orally-challenged (but non-vaccinated) mice (Table 2B). Overall, vaccination with Regimens I and II successfully elicited humoral and cell-mediated systemic immune responses to a *Cryptosporidium*-derived antigen in both nourished and malnourished mice, and these responses were superior to those generated by oral challenge with *C. parvum* oocysts alone ('natural infection'). Of note, strong systemic IFN- γ secretion was found after vaccination, even in the setting of prolonged malnutrition.

3.3. Impact of Cp15 vaccination on resistance to *C. parvum* infection

The findings above in nourished and malnourished mice using intra-nasal priming suggested that vaccination with Cp15 might improve disease outcome of the host when challenged with an infectious dose of *C. parvum* oocysts. Therefore, groups of mice, administered Regimen I or II, were challenged by gavage with oocysts at 10–20 days following the final dose of vaccine, and accessed for growth and stool shedding. For mice receiving Regimen I, vaccination did not enhance body weight gain of nourished *or* malnourished mice after challenge with *C. parvum*, compared to infected-only controls over a 12 day period following oocyst challenge (Fig. 1A). Stool shedding was quantified by PCR next as a measure of intensity of infection. In *nourished* mice administered Regimen I, stool shedding of parasites diminished to only a few organisms after day 5, with or without vaccination (Fig 1B). For *malnourished* mice administered vaccine Regimen I, stool shedding of organisms improved transiently (days 5 and 7, $p < 0.05$), but was not otherwise different from non-vaccinated oocyst-challenged controls (days 1, 3, 9 and 11, $p>0.12$, Fig 1B). Malnourished infected mice, compared to nourished infected mice were observed to have greater shedding (by > 2 logs, $p<0.05$, days 5–7 post-challenge, Fig 1B), irrespective of vaccination status when administered Regimen I. Similarly, Regimen II, consisting of vaccination of nourished mice with Cp15 including injection of antigen in alum together with pre- and post-challenge administration of CpG and alanyl-glutamine, did not significantly improve the growth rate

of mice that were subsequently malnourished then challenged (Fig 2A). Further, Regimen II did not reduce stool shedding of parasites when compared to that of infected only control mice ($p>0.19$, days 5 & 7, Figure 2B).

In summary, these findings indicate that, while Cp15-directed vaccination induces a potent humoral and cell-mediated systemic immune response in the malnourished mouse, it had, at best, a modest impact on stool shedding (Fig 1B, days 5&7). Importantly, the host's ability to generate IFN- γ , known to be crucial to successful eradication of *Cryptosporidium* [7], was preserved and quite robust in the malnourished Cp15-vaccinated host.

3.4 Local mucosal events following vaccination

In addition to eliciting a *systemic* response to a candidate *Cryptosporidium*-derived vaccinogen, a regimen which includes intra-nasal antigen priming might generate *local mucosal* immune responses in the nourished and/or malnourished host. Therefore, mesenteric lymph nodes were harvested from mice administered Regimen I twenty days after the final dose of vaccine, and examined for cytokine secretion in response to Cp15 (Table 3). In *nourished vaccinated (but non-infected) mice*, responses were observed for IL-6 and IFN- γ , similar to those found with Study 2 splenocytes (2041 versus 1702 for IL-6; 3956 versus 3480 for IFN- γ). IL-2 and IL-10 secretion was minimal. *For malnourished vaccinated (but non-infected) mice*, IFN- γ secretion was less but clearly evident (Table 3) and IL-6 secretion was reduced. These data suggest that immunization regimens, initiated by intra-nasal priming with a candidate vaccinogen expressed by a live enteric bacterial vector, can generate a local vaccinogen-specific response in mesenteric lymph nodes in which IFN- γ secretion is substantial but moderately dependent on the nutritional status of the host.

3.5. Antibody to LPS following vaccination with an enteric bacterial vector

Earlier reports suggested that the presence of circulating immunoglobulin to LPS after exposure to a live attenuated enteric organism could indicate immunological priming and/or disruption of the intestinal barrier [25]. To determine if either occurred following administration of the enteric bacterial vector in the current study, anti-LPS titers were determined at the time of euthanasia 20–40 days after vaccination. Titers of anti-LPS exceeding 1:6000 were consistently detected in mice administered the *Salmonella*-based live bacterial vaccine vector, while titers found in non-vaccinated mice were $< 1:400$, irrespective of the nutritional status of the host ($p<0.01$). These findings suggest that intra-nasal exposure to *Salmonella enterica* Serovar 908 htrA may lead to immunological priming of the host, as well as to intestinal barrier disruption sufficient to elicit anti-LPS antibody.

4.0. Discussion

Malnutrition, prevalent in the developing world especially among young children, has been cited as an important factor underlying limited efficacy of vaccines in those areas [13]. Investigations of the biological basis for the effect of malnutrition on vaccine responses have revealed, in some cases, a diminished immune/inflammatory reactivity to antigens present in the vaccine [13], and a reduced dendritic cell function/maturity in neonatal models, partially reversible with administration of the cytokine TNF- α [26].

Our findings herein using a new freshly weaned mouse model, vulnerable to infection with *C. parvum*, suggest that malnutrition alone can blunt antigen-specific cell-mediated responses to a vaccinogen (Table 3), as measured in *mesenteric* lymph node cells of the gut-associated lymphoid system, crucial to host resistance to enteric infection. That is, in antigen-specific recall assays, we observed reduced secretion of IL-6 and IFN- γ , and this may underlie the diminished resistance to *C. parvum* oocyst challenge found in models that incorporate malnutrition. On the other hand, systemic responses assessed *by splenocyte*

cytokine secretion demonstrated generally preserved IFN- γ and IL-6 responses (Table 2C) in the setting of malnutrition, including higher IFN- γ in some cases. Anti-Cp15 serum titers remained strong. Thus, the regimen of intra-nasal priming and parenteral boosts studied here in malnourished mice, generated a quantifiable antigen-specific *systemic* humoral and cell-mediated response that rivaled that in nourished mice, and did not exhibit the blunted immune response that was anticipated with prolonged malnutrition.

As an alternative to only oral or parenteral vaccine administration (27,28), several studies have incorporated intra-nasal immunization into a heterologous prime-boost vaccine approach, consisting of mucosal priming in the nares with a *Salmonella enterica* typhi strain expressing and exporting the vaccinogen fused to cytolysin A (ClyA), followed by a parenteral (i.p., i.m., or i.d.) boost of the vaccinogen in adjuvant [17–19]. Success of this approach is enhanced by the vector's expression of random repeats on the surface of the bacterium; by its ability to release study vaccinogen as facilitated by fusion to an auto-transporter; as well as by its extended presence intra-lumenally for eliciting a potent humoral and cell-mediated mucosal immune response [17,18,29,30].

Previous work by Manque *et al.* [19] showed that a vaccination protocol involving both live vector priming followed by parenteral boosts, similar to but differing from Regimen I of the current study (paragraph 1, Supplementary Material), using Cp15 and at least one other *Cryptosporidium* antigen, induces strong cellular and humoral immune responses including IFN- γ and IL-6. However, we are unaware of reports of cytokine secretion in *malnourished weaned or adult mice* administered a prime-boost regimen, i.e., intra-nasal live bacterial vector expressing a *Cryptosporidium*-derived antigen followed by a parenteral antigen boost. Further, Guk, Young, Chai [31] explored which compartment of T lymphocytes in the small intestine of four week old C57BL/6 mice might be responsible for cytokine secretion in response to *C. parvum*. Ten days after an oocyst oral challenge, the proportion of lamina propria lymphocytes (LPL) expressing IFN- γ and TNF- α (but not IL-2) was significantly increased, while there was little alteration in cytokine secretion by intra-epithelial lymphocytes (IEL). These findings suggested LPL but not IEL play an important role in the primary T lymphocyte response to *C. parvum* [32].

The capacity of two prime-boost vaccine regimens (I & II) to elicit robust *antibody* titers to a *Cryptosporidium*-derived antigen in nourished and malnourished mice is noteworthy. Human volunteers with pre-existing anti-*C. parvum* serum IgG subsequently challenged with oocysts had an ID₅₀ > 20 times that in sero-negative volunteers [33]. At least one study reported immunoglobulin to *C. parvum* antigen given orally within two hours of exposure was protective [34], potentially interrupting attachment of the organism to epithelium, independent of the cell-mediated immune response in the exposed host.

In the current study, despite prolonged malnutrition of the host, IFN- γ secretion was unexpectedly preserved systemically although moderately (50%) diminished locally in mesenteric lymph node cells. This would suggest that the malnourished mouse was able to sustain, both systemically and to a lesser extent mucosally, this important component of the immune response for limiting *C. parvum* infection. While identity of the cell type(s) that retains the capacity for secretion of IFN- γ despite prolonged malnutrition was not explored, the study by Guk *et al.* (above) suggests that it may reside in the LPL, where it may be less susceptible to functional deficits resulting from malnutrition.

Neither of the two prime-boost regimes in the current study was able to consistently protect the host against weight loss and robust stool shedding after challenge with $2\text{--}5 \times 10^7$ excysted or unexcysted *C. parvum* oocysts (Fig 1A/B and 2A/B). Given the preservation of IFN- γ production systemically, and only a moderate local (mucosal) decrement of IFN- γ

secretion, other mechanisms may be in play to explain the vaccine's limited efficacy in reducing growth shortfalls and stool shedding in the model.

Limitations of our study are several. A dose-response study with carefully graded reductions in the *C. parvum* oocyst dose used to challenge mice was not performed, and may have uncovered an impact of vaccination on stool shedding and body weight change when the host is confronted by a smaller parasite challenge. As indicated above, though, the important parameter of weight loss in the mouse model is not evident at challenge doses of $< 2 \times 10^7$ oocysts/mouse, making meaningful evaluation of vaccine efficacy more difficult. Secondly, we did not measure, as an outcome of the *in vivo* studies of vaccine regimens, fecal IgA, either total or *C. parvum*-specific---a potentially important component of a protective local mucosal immune response [28]. However, studies showing IgA administration to infected persons enhances host resistance to *C. parvum* infection are not reported to our knowledge.

In summary, intra-nasal prime-boost regimens against a *Cryptosporidium*-derived antigen expressed by a live *Salmonella* vector demonstrated immunogenicity, both systemically (in spleen) and locally (in mesenteric lymph nodes) in the nourished and in the malnourished host. Secretion of IFN- γ , central to resistance to this organism, was prominent among cytokines secreted by lymphocytes. Thus, an intra-nasal prime-boost vaccine regimen is now available for eliciting a sustained systemic as well as a local response to specific *Cryptosporidial* antigens, irrespective of the nutritional status of the host. Application of this methodology to putative disease-protective antigens such as a calcium-activated apyrase for cryptosporidiosis (35), may be indicated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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HIGHLIGHTS

1. For cryptosporidiosis causing acute/chronic diarrhea, there is no effective vaccine.
2. We tested a vaccine based on gene sequence and priming with an intra-nasal live vector.
3. In vaccinated nourished mice, IL-6, IFN- γ and antigen-specific Ig were robust.
4. In vaccinated malnourished mice, systemic (not local) immunity was undiminished
5. A vaccine regimen applicable to humans irrespective of nutritional status is elucidated.

Figure 1A

Figure 1B

Figure 1.

Figure 1A Effect of a live enteric bacterial Cp15 vaccine on weight change in parasite-challenged nourished or malnourished C57BL/6 mice. Mice were began on defined diets on day 24 of life, and vaccinated with prime-boost regimen I (Study 2, Table 1). Both vaccinated and non-vaccinated mice were challenged with 5×10^7 unexcysted *C. parvum* oocysts/mouse by gavage at 58 days of life. No significant differences were found within the nourished or within the malnourished groups. N = 6 per group.

Figure 1B. Shedding of parasites in stool from mice described in Figure 1A, as influenced by the vaccination and nutritional status. Shown are the mean number of parasites quantified in duplicate by pcr and normalized per mg of stool, determined the day following parasite

challenge (day 1) and assessed through day 11. * designates $P < 0.01$, and **, $P < 0.05$, comparing shedding in malnourished mice that underwent vaccination with those that did not. N = 6/group.

Figure 2A

Figure 2B

Figure 2.

Figure 2A Impact of Regimen II on body weight change in C57BL/6 mice after challenge with unexcysted *C. parvum* oocysts. Mice were fed with a regular (20%) protein diet during the immunization phase through day 59, then malnourished (2% protein diet) for 14 days prior challenge with 2×10^7 unexcysted oocysts/mouse; non-vaccinated mice underwent oocyst challenge at the same time (day 73 of life). N = 8/group.

Figure 2B. Stool shedding of *C parvum* parasites in mice described in Figure 2A who were administered Regimen II and in control mice (infected only). Quantitative pcr was used to determine in duplicate the number of parasites in stool samples, and this is expressed as the mean number per mg of stool. There were no statistically significant differences between the vaccinated and non-vaccinated groups, assessed on the day after challenge (day 1) through day 7. N = 8/group.

Table 1
Summary of vaccination study protocols to evaluate two immunization regimens I and II.

	Diet	Prime	Boost # 1	Event	Boost # 2	Challenge****
Study 1 * Regimen 1	Change diet at 24 days of life	i.n. Cp15 live vector at 28 days of life	i.p. Cp15 antigen with complete Freund's adjuvant at 38 days of life	i.p. Cp15 antigen with complete Freund's adjuvant at 38 days of life	i.p. Cp15 antigen with incomplete Freund's adjuvant at 48 days of life	5×10 ⁷ excysted <i>C. parvum</i> oocysts/mouse (non-vaccinated groups only) at day 28 of life)
Study 2 ** Regimen 1	Change diet at 24 days of life	i.n. Cp15 live vector at 28 days of life	i.p. Cp15 antigen with complete Freund's adjuvant at 38 days of life	i.p. Cp15 antigen with complete Freund's adjuvant at 38 days of life	i.p. Cp15 antigen with incomplete Freund's adjuvant at 48 days of life	5×10 ⁷ unexcysted <i>C. parvum</i> oocysts/mouse at 58 days of life
Study 3 *** Regimen 2	Change diet at day 59 of life	i.n. Cp15 live vector at 29 days of life	i.n. Cp15 live vector at 42 days of life	i.n. Cp15 antigen with Alum at 56 days of life	i.m. Cp15 antigen with Alum at 56 days of life	2×10 ⁷ unexcysted <i>C. parvum</i> oocysts/mouse at 73 days of life

* Study 1 using Regimen I compares immunogenicity of vaccine and natural infection in malnourished and nourished mice. Only non-vaccinated mice were challenged with 5×10⁷ excysted *C. parvum* oocysts/mouse at 28 days of life.

** Study 2 using Regimen I investigates vaccine efficacy in nourished and malnourished vaccinated mice for protection against *C. parvum* challenge.

*** Study 3 assessed the efficacy of a second immunization scheme (Regimen II) consisting of two intra-nasal exposures to live vector followed by one boost with antigen in Alum. Mice were given a normal diet during the immunization phase and then malnourished beginning at 59 days of life prior to challenge with *C. parvum* oocysts.

**** Specimens for cytokine measurements were taken at the time of euthanasia, 20–40 days followed *C. parvum* challenge; the later occurred on day 28 (Study 1), day 58 (Study 2), and day 73 (Study 3) of life.

Table 2

Impact of nutritional status on immune responses to immunization Regimens I and II, and to oral challenge with *C. parvum* oocysts only.

A. Nourished, vaccinated mice

Study			
Cytokine (pg/ml, mean±1SD) and Ig to Cp15 **	1 *	2	3
IL-2	368	62±55	ND
IL-6	5206	1473±685	ND
IFN- γ	10708	3000±1493	ND
Serum IgG to Cp15	1:25,600	> 1:25,600	ND

B. Nourished, orally challenged (not vaccinated) mice.

Study			
Cytokine(pg/ml, mean ±1 SD) and Ig to Cp15 **	1 *	2	3
IL-2	39	4±3.7	ND
IL-6	1903	524±324	ND
IFN- γ	537	2796±384	ND
Serum IgG to Cp15	1:1,600	1:466±149	ND

C. Malnourished, vaccinated mice

Study			
Cytokine(pg/ml, mean±1SD) and IgG to Cp15 **	1 *	2	3
IL-2	311	87±133	732±703 ***
IL-6	1695	1269±714	2244±1377 ***
IFN- γ	13762	3250±1864	3076±927 ***
Serum IgG to Cp15	1:6,400	> 1:25,600	1:5403±35

D. Malnourished, orally challenged (not vaccinated) mice

Study			
Cytokine (pg/ml, mean±1SD) and IgG to Cp15 **	1 *	2	3
IL-2	19	3.6±3.4	1551
IL-6	672	300±92	1492
IFN- γ	116	154±103	1552
Serum IgG to Cp15	1:800	1:2240±2110	1:1200±1164

* Specimens pooled by treatment group prior to assay. ND, not done.

** Serum IgG to Cp15 was determined using samples of individual mice in Studies 2 & 3; that in Study 1 used a pooled specimen.

*** Calculated values (see Statistical Analysis, [Methods and Materials](#)).

Table 3

Cytokine secretion by mesenteric lymph node cells in response to study antigen Cp15.

Cytokine (pg/ml, mean $\pm$ 1SD) *	Diet	
	Nourished	Malnourished
IL-2	<20	115
IL-6	1866	296
IFN- γ	3956	2081
IL-10	170	<10

* Specimens pooled prior to analysis