Re-feeding rapidly restores protection against *Heligmosomoides bakeri* (Nematoda) in protein-deficient mice

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SUMMARY

This study determined whether the timing of re-feeding of protein-deficient mice restored functional protection against the gastrointestinal nematode, *Heligmosomoides bakeri*. Balb/c mice were fed a 3% protein-deficient (PD) diet and then transferred to 24% protein-sufficient (PS) diet either on the day of primary infection, 10 days after the primary infection, on the day of challenge infection, or 7 days after the challenge infection. Control mice were fed either the PD or PS diet. Onset of challenge, but not primary, infection caused short-term body weight loss, anorexia and reduced feed efficiency. Weight gain was delayed in mice when re-feeding commenced on the day of challenge infection; alkaline phosphatase (ALP) was also elevated in these mice on day 28 post-challenge. In contrast, other re-feeding groups attained similar body weights to PS mice within 4 days and had similar ALP at day 28. Serum leptin was higher in PD than PS mice and positively associated with food intake. As expected, worm survival was prolonged in mice fed the PD diet. However, egg production and worm burdens were similar in all re-feeding groups to the PS mice, indicating that protein re-feeding during either the primary or challenge infection rapidly restored normal parasite clearance.

Key words: protein deficiency, protein re-feeding, nutritional status, mouse, nematode, *Heligmosomoides bakeri*, resistance, infection-induced weight loss, anorexia.

INTRODUCTION

Clearance of *Heligmosomoides bakeri* following a challenge infection in well-nourished mice is understood to involve both parasite-specific immunological responses (Urban *et al*. 1991*a*, *b*) and inflammatory responses in the gut (Shea-Donohue *et al*. 2001), orchestrated primarily by the Th2 cytokines IL-4 and IL-13 (Urban *et al*. 1991*b*; Gause *et al*. 2003). The early stage of infection (L_4) is relatively protected while it is embedded in the serosal musculature although its development and the timing of emergence into the gastrointestinal (GI) lumen are delayed during a challenge infection (Ey, 1988). It is only once the adult parasites are in the lumen, however, that effector responses contribute to worm expulsion. Enhanced intestinal epithelial cell ion flux (Shea-Donohue *et al*. 2001), increased mucosal permeability (Madden *et al*. 2002) and increased intestinal smooth muscle contractility (Zhao *et al*. 2003), together with Th2 immunological responses (Gause *et al*. 2003) are believed to induce expulsion of *H. bakeri* from the GI tract. Parasite clearance presumably depends, therefore, on initial priming of the immune response during the first exposure to the parasite and rapid development of both immunological and inflammatory effector responses during challenge so that both are fully functional when the adults emerge into the GI lumen.

Protein energy malnutrition (PEM) often coexists with GI nematode infections (Koski and Scott, 2001) because infections cause malnutrition and PEM increases susceptibility to infection (Scrimshaw and San Giovanni, 1997; Koski and Scott, 2001). Immunity is considered to be a crucial causal link as both human and laboratory studies have demonstrated that PEM has wide-spread effects on both systemic and gut-associated lymphoid tissues, including reduced homing of mucosal lymphocytes to the gut (McDermott *et al*. 1982), reduced activity of natural killer cells (Ingram *et al*. 1995), reduced release of secretory IgA (Ha and Woodward, 1997), reduced cytokine production by both Th1 (Doherty *et al*. 1994) and Th2 cells (Ing *et al*. 2000) and reduced numbers of memory cells (Najera *et al*. 2001). At the level of the gut, protein deficiency alone induces villus and mesenteric lymph node (MLN) atrophy and crypt hypoplasia (Bell *et al*. 1976; Syme, 1982; Woodward and Miller, 1991; Deitch *et al*. 1992), decreases numbers of B lymphocytes (Bell *et al*.

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Table 1. Composition of experimental diets (g/kg diet)

Ingredient	Protein-sufficient diet (24%)	Protein-deficient diet (3%)
Egg white solids[1]	240	30
Corn oil[2]	80	80
Corn starch[2]	296	506
Glucose [2]	296	296
Cellulose (Alphacel)[2]	29·5	29·5
Vitamin mix[3]	12	12
Mineral mix[4]	46·5	46·5

[1] Ovalbumin (Dried Egg White Solids Type P-110), ICN Biochemicals Canada Ltd, Montreal, PQ.
[2] ICN Biochemicals.
[3] Vitamin mix (mg/kg diet): vitamin A acetate 8·0; cholecalciferol 0·025; α-tocopheryl acetate 60; menadione 1·15; biotin 3·6; folic acid 2·0; nicotinic acid 50·0; *d*-calcium pantothenate 32·0; riboflavin 24·0; thiamin hydrochloride 16·0; pyridoxine hydrochloride 4·0; cyanocobalamin 0·05; butylated hydroxytoluene 100; choline chloride 4000; alphacel 7699·175.
[4] Mineral mix (mg/kg diet): $CaHPO_4$ 27·16; $KHCO_3$ 10·25; NaCl 2·54; $MgSO_4$ 4·95; $CrK(SO_4)_2 \cdot 12H_2O$ 0·0384; $CuCO_3 \cdot Cu(OH)$ 0·0521; KIO_3 0·001; $FeSO_4 \cdot 7H_2O$ 0·87115; $MnCO_3$ 0·10462; $ZnCO_3$ 0·09588; Na_2SeO_3 0·0004; $Na_2MoO_4 \cdot 2H_2O$ 0·000378; $KF \cdot 2H_2O$ 0·0099; citric acid 0·42617.

1976) and CD4+ T cells (Woodward *et al*. 1999), reduces mast cell and goblet cell responses (Wells, 1962, 1963; Madi *et al*. 1970), increases permeability of the gut mucosal barrier (Cummins *et al*. 1987*a*, *b*; Deitch *et al*. 1992), and induces oxidative stress by increasing catalase activity and the concentration of thiobarbituric acid-reacting substances (Darmon, 1993) leading to intestinal dysfunction. Importantly, protein deficiency also prolongs survival of *Heligmosomoides bakeri* (=*Heligmosomoides polygyrus*, *Nematospiroides dubius*) (Slater and Keymer, 1988; Boulay *et al*. 1998; Ing *et al*. 2000), a murine nematode commonly used as a model for human and livestock GI nematode infections.

We have previously demonstrated (Ing *et al*. 2000) that protein deficiency blocks the protective response to *H. bakeri*, but how quickly protective responses are restored following protein re-feeding was not described. Protein re-feeding of uninfected rats for only 3 days has been shown to replete body weight, serum albumin, insulin-like growth factor 1, leucine concentrations and colon structure (Qu *et al*. 1996). Re-feeding also elevates crypt cell mitotic index in the mouse duodenum (Acra *et al*. 1998), rapidly restores non-specific inflammatory effectors in mice (Ikeda *et al*. 2001) and activity of intestinal enzymes in rhesus monkeys (Rana *et al*. 1995, 2003). However, the rapidity of restoration of immune responses to fungal infection has been shown to depend on the relative timing of infection and re-feeding (Oarada *et al*. 2002).

The objective of the present study was to determine whether the timing of protein re-feeding influenced restoration of nutritional status and functional protection against the GI nematode, *H. bakeri*, in protein-deficient mice. We hypothesized that the presence of infection would prolong restoration of nutritional status. We further hypothesized that functional protection during the challenge infection might be impaired if protein deficiency extended throughout the period of primary infection and into the challenge infection, but that functional protection would be restored if re-feeding occurred prior to the challenge infection.

MATERIALS AND METHODS

Experimental design

To establish if protein re-feeding restored functional protective immunity against *H. bakeri*, nutritional, parasitological and immunological indicators measured in control mice, fed either a protein sufficient (PS) diet or a protein-deficient (PD) diet throughout the study, were compared with those of 4 groups of mice fed a PD diet for varying periods of time before they were transferred to a PS diet. Re-feeding (RF) commenced on the day of primary infection (RF0Pr), 10 days after the primary infection (RF10Pr), on the day of challenge infection (RF0Ch), and 7 days after challenge infection (RF7Ch).

Diets and mice

Two egg-white based semi-purified powdered diets were formulated (Table 1). In the 24% PS diet, energy from protein made up 24% of total calorie intake, an amount sufficient for maintenance of normal physiology, metabolism and growth of mice (NRC, 1995). In contrast, the PD diet contained 3% protein; previous experiments in our lab have shown that mice fed this diet have reduced body weight gain, prolonged *H. bakeri* survival and suppressed Th2 immune responses (Boulay *et al*. 1998; Ing *et al*. 2000). Both diets were identical for all nutrients except the concentrations of protein and carbohydrate, the latter of which was adjusted to ensure that both diets were isocaloric. The diet formulation was based on National Research Council mouse requirements (NRC, 1995) and ensured that a 30% reduction in food intake due to infection or protein deficiency would not generate other nutrient deficiencies.

Sixty, 3-week-old female BALB/c mice (Charles River Breeding Laboratories, Quebec, Canada) were housed individually in Nalgene cages (Fisher Scientific, Montreal, Canada) with stainless steel covers in a temperature controlled mouse room (22–25 °C) with a 14 h:10 h light:dark cycle. Food and tap water were provided to the mice *ad libitum*.

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All mice were fed mouse chow (Mouse Chow 5015, Agribands Canada, Ontario, Canada) for 3 days and then acclimatized for 4 days to the PS diet provided in mouse Powder Feeders (Lab Products, Quebec, Canada) especially designed to reduce spillage of powdered diets. Then the mice were randomly assigned to one of the 6 diet groups (10 mice per group). PS and PD mice were fed their respective diets throughout the experiment, beginning 7 days before infection. All mice in the 4 re-feeding groups were fed the PD diet beginning 7 days before infection until the day of re-feeding, at which time they were fed the PS diet until the end of the study.

Infection protocol

H. bakeri maintained in outbred CD-1 mice (Charles River) were used as the source of infective third-stage larvae (L_3). Mice were infected with 100 ± 4 L_3 by oral gavage, and the accuracy of the infection dose was determined by counting 10 sham doses. A standard anthelmintic-abbreviated immunizing protocol was used (Behnke and Robinson, 1985) whereby the primary infection was drug-terminated with pyrantel pamoate (172 mg/kg body weight; Pfizer Canada Inc., Montreal, Canada) on days 9 and 14 after the primary infection; mice were then challenged with 100 ± 4 L_3 one week later (21 days after the primary infection). In well-nourished mice, parasites are normally expelled within 1 month post-challenge infection (p.c.i.) (Behnke and Robinson, 1985). On days 16 and 26 p.c.i., 24 h egg production was measured from individual mice, using a modified McMaster technique (Scott, 1988). Mice were killed on day 28 p.c.i. using Ketamine/Xylazine (50/5 mg/kg body weight) anaesthesia. Blood was collected from the tail vein (day 10 p.c.i.) or cardiac puncture (day 28 p.c.i.) and serum samples were stored at -20 °C for later detection of total serum IgE, leptin, and alkaline phosphatases (ALP). All procedures were approved by the McGill's Macdonald Campus Animal Care Committee according to the Canadian Council on Animal Care Guidelines and Policies.

Nutritional status indicators

Body weight, food intake and feed efficiency (weight gain per day/food intake per day) were recorded every 2–3 days. For each diet group, weight gain and cumulative food intake were calculated for the 4 days prior to, and after, both primary and challenge infection, and the 4 days prior to, and after, commencement of re-feeding.

Serum leptin was assayed at day 28 p.c.i., using a mouse leptin ELISA kit (Linco Research, Missouri, USA, Cat. EZML-82K), according to the manufacturer's instructions. Briefly, 10 μl of standards and samples were added to mouse leptin microtitre plates which had been pre-coated with pre-titred capture antibodies. Then 50 μl of pre-titred anti-rodent leptin was added to each well and the 96-well plates were then incubated for 2 h. Then 100 μl of pre-titred biotinylated anti-mouse leptin antibody was added to each well, wells were incubated for 1 h, 100 μl of pre-titred streptavidin-horseradish peroxidase conjugate was added and wells were incubated for a further 30 min. The substrate 3,3′,5,5′-tetramethylbenzidine (TMB) was then added and incubated for 30 min and the reaction was stopped by adding 0·3 M hydrochloric acid. The plates were read with an EL 309 Microplate Autoreader (Bio-Tek Instruments, Inc. Winooski, Vermont) at 450 nm and 590 nm. The OD value was calculated by subtracting the reading at 590 nm from the reading at 450 nm and averaged across the duplicates for each sample.

Parasitological indicators

Worm fecundity was estimated by dividing 24 h egg release per mouse on day 26 p.c.i. by the total number of female worms on day 28 p.c.i. At necropsy, the small intestines were excised and the numbers of adult worms in the intestine were counted and sexed.

Indicators of immunity and tissue damage

Total serum IgE was measured at both days 10 and 28 p.c.i., by a two-site ELISA following the manufacturer's directions (BD PharMingen, Mississauga, Ontario). Immulon II plates (Becton Dickinson and Co., Oxnard, Ontario) were coated with rat anti-mouse IgE mAb (R35–72, BD PharMingen) and developed with biotinylated rat anti-IgE mAb (R35–92, BD PharMingen) and avidin-peroxidase (BD PharMingen). TMB was used as a substrate. After stopping the reaction with phosphoric acid, the plates were read with a Microplate Autoreader (EL309, Mandel Scientific Company Ltd, Guelph Ontario) at 450 nm. IgE concentration was computed according to an IgE standard curve with known concentrations of mouse IgE standard (C38-2, BD PharMingen) and the average of duplicate values is reported as μg/ml of serum.

Alkaline phosphatase concentrations (ALP) in serum were measured (Mulivor *et al*. 1985) as an index of tissue damage. Total serum ALP was assayed at days 10 and 28 p.c.i., and serum concentrations of the intestinal and liver-kidney-bone (LBK) isoenzymes were assayed on day 28 p.c.i. All assays were done in triplicate, using the Promega AttoPhos® AP Fluorescent Substrate System (Promega; Madison, Wisconsin), according to the manufacturer's protocol. Absorbance readings were taken using the Wallac fluorimeter (Turku, Finland), set at 430–440 nm for the excitation filter and 550–560 nm for the emission filter.

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Table 2. Impact of re-feeding with protein sufficient diet on nutritional, parasitological and immunological outcomes (mean ± S.E.)[1,2]

	PS	RF0Pr	RF10Pr	RF0Ch	RF7Ch	PD
Nutritional outcomes						
Food intake, g/d	2·84 ± 0·05^{ab}	2·72 ± 0·05^{a}	2·77 ± 0·04^{ab}	2·83 ± 0·03^{ab}	3·01 ± 0·06^{b}	2·80 ± 0·08^{ab}
Food intake between day of primary infection and of re-feeding, g/d[3]	—	—	2·98 ± 0·07^{ab}	3·13 ± 0·03^{bc}	3·28 ± 0·08^{c}	—
Food intake after protein re-feeding, g/d	—	2·72 ± 0·05	2·72 ± 0·04	2·60 ± 0·05	2·66 ± 0·03	—
Total weight gain, g	3·62 ± 0·16^{a}	3·63 ± 0·31^{a}	3·83 ± 0·24^{a}	3·25 ± 0·33^{a}	3·61 ± 0·22^{a}	−1·35 ± 0·58^{b}
Time to attain similar body weight to PS group, d	—	0	4	13	4	—
Serum leptin, day 28 p.c.i.[4], ng/ml	4·4 ± 0·3	4·9 ± 1·0	5·5 ± 0·6	5·4 ± 0·7	4·0 ± 0·4	5·9 ± 0·6
Parasitological outcomes						
Net egg production/d						
day 16 p.c.i.	92 ± 32^{a}	193 ± 118^{a}	160 ± 51^{a}	103 ± 59^{a}	159 ± 77^{a}	4676 ± 1193^{b}
day 26 p.c.i.	25 ± 13^{a}	84 ± 45^{a}	311 ± 274^{a}	9 ± 9^{a}	233 ± 103^{a}	10 668 ± 1691^{b}
Number of worms day 28 p.c.i.	2·4 ± 0·6^{a}	3·7 ± 1·0^{a}	5·4 ± 1·5^{a}	3·3 ± 0·6^{a}	3·8 ± 0·9^{a}	43·8 ± 2·8^{b}
Fecundity, eggs/female worm/d day 26 p.c.i.	10 ± 7^{a}	80 ± 41^{a}	51 ± 34^{a}	5 ± 5^{a}	152 ± 50^{a}	449 ± 45^{b}
Immunological outcomes						
Total serum IgE, μg/ml						
day 10 p.c.i.[5]	95 ± 10^{ab}	90 ± 10^{ab}	62 ± 10^{a}	79 ± 10^{ab}	86 ± 9^{ab}	118 ± 11^{b}
day 28 p.c.i.	51 ± 6	54 ± 5	55 ± 4	51 ± 3	44 ± 5	53 ± 4

[1] PS mice fed protein sufficient diet throughout the experiment; RF0Pr mice fed protein deficient diet, then fed PS diet beginning on day 0 of primary infection; RF10Pr mice fed protein deficient diet, then fed protein sufficient diet beginning on day 10 of primary infection; RF0Ch mice fed protein deficient diet, then fed protein sufficient diet beginning on day 0 of challenge infection; RF7Ch mice fed protein deficient diet, then fed the protein sufficient diet beginning on day 7 of challenge infection; PD mice fed the protein deficient diet throughout the study.
[2] Different lower case letters across a row indicate significant differences among experimental groups as revealed by *post-hoc* tests ($P<0·05$).
[3] Average food intake between day of primary infection and day of re-feeding significantly higher than average food intake after protein re-feeding ($P<0·001$).
[4] Post-challenge infection.
[5] Serum IgE is significantly higher at day 10 p.c.i. than at day 28 p.c.i. ($P=0·03$).

Statistics

All results were expressed as mean ± S.E.M. Body weight and food intake data were initially analysed by repeated measures ANOVA, and the change in weight and food intake during the 4 days before and after infection or re-feeding was subsequently analysed by one-way ANOVA. Data on parasite reproduction and worm burdens were analysed using Kruskal Wallis non-parameteric ANOVA. IgE, ALP and leptin were analysed using one-way or two-way ANOVA depending on whether the effect of time was included. Bonferroni or Scheffe's test was performed when one-way ANOVA revealed a significant main effect. Finally, regression analysis was used to determine whether there was a linear relationship between ALP or leptin and worm numbers. All data were analysed by SAS 9.01 (SAS Institute, Cary, NC) and $P<0·05$ was considered as significant.

RESULTS

Infection

Egg production (day 16 p.c.i.) was elevated in the PD mice, but was very low in PS mice, and also in mice from all of the re-feeding groups (Table 2). A similar result was observed for egg production at day 26 p.c.i., and for worm burdens and *per capita* fecundity at day 26 p.c.i. (Table 2). Together these results demonstrate that re-feeding even as late as 7 days after the challenge infection restored the functional protection that had been impaired by protein deficiency.

Serum samples collected at days 10 and 28 p.c.i. were assayed for total IgE as a systemic index of immune response against *H. bakeri*. Although IgE did not differ between PS and PD mice at either time-point, suggesting that total IgE was not perturbed by PD at these times, serum IgE was higher at day 10 p.c.i., compared with day 28 p.c.i. (Table 2);

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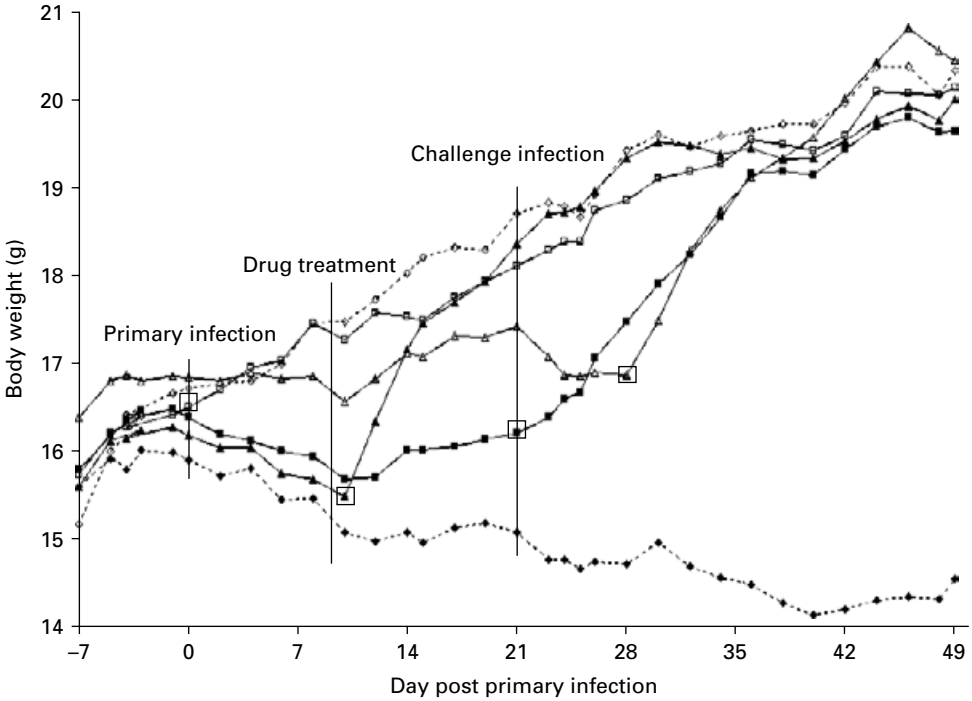

Fig. 1. Body weight over time in mice infected with *Heligmosomoides bakeri* on day 0, and again on day 21 following drug treatment on days 9 and 14. The time of primary infection, the first drug treatment and the challenge infection are indicated by the vertical lines. PS mice (- -◇- -) fed protein sufficient diet throughout the experiment; RF0Pr mice (—□—) fed protein deficient diet, then fed protein sufficient diet beginning on day 0 of primary infection; RF10Pr mice (—▲—) protein deficient diet, then fed protein sufficient diet beginning on day 10 of primary infection; RF0Ch mice (—■—) fed protein deficient diet, then fed protein sufficient diet beginning on day 0 of challenge infection; RF7Ch mice (—△—) mice fed the protein deficient diet, then fed the protein sufficient diet beginning on day 7 of challenge infection; PD mice (- -◆- -) fed the protein deficient diet throughout the study. The time of re-feeding is indicated by the square boxes.

also the highest IgE concentration was obtained in PD mice and the lowest was found in the RF10Pr group.

Body weight

Body weights during the pre-feeding week were similar among all diet groups. However, by day 6 post-infection (p.i.), the negative impact of protein deficiency on weight gain was evident (Fig. 1). By the end of the experiment, PD mice had lost body weight; however, mice in all re-feeding groups had regained their lost weight and had weights similar to PS mice (Fig. 1; Table 2). No catch-up growth occurred in the RF0Pr group (Fig. 2A), as body weights did not differ from those of PS mice at the time they were re-fed (Fig. 1). However, re-feeding induced rapid weight gain in RF10Pr and RF7Ch mice (Fig. 2A) and body weight was similar to that of the PS group within 4 days of re-feeding (Table 2). In contrast, in the case of the group re-fed on the same day as the challenge infection (RF0Ch), no significant improvement in weight (Fig. 2a) occurred over the 4 days following re-feeding, and full restoration of body weight did not occur until 13 days after re-feeding (Fig. 1; Table 2), indicating that the stress of larval challenge on the day of re-feeding interfered with the host's ability to benefit from the PS diet.

Food intake and feed efficiency

In order to determine whether the delayed weight gain in the RF0Ch mice may have resulted from the stress associated with the larval challenge, we compared both body weight change and feed efficiency during the 4 days prior to infection with data from the 4 days immediately after infection in both PS and PD groups. Food intake (Fig. 2D) and feed efficiency (data not shown) were significantly reduced immediately following challenge infection in all groups of mice, indicating that larval challenge induced anorexia. The challenge infection also caused PS mice to stop gaining weight, and caused significant weight loss in PD mice (Fig. 3B). Interestingly, food intake immediately after the primary infection was also reduced in PS mice, whereas food intake increased significantly in 3 of the 4 groups continuing to consume the PD diet (RF10Pr, RF0Ch, RF7Ch) (Fig. 2C). Also, the concentration of serum leptin on day 28 increased linearly with cumulative food intake during the last week of the

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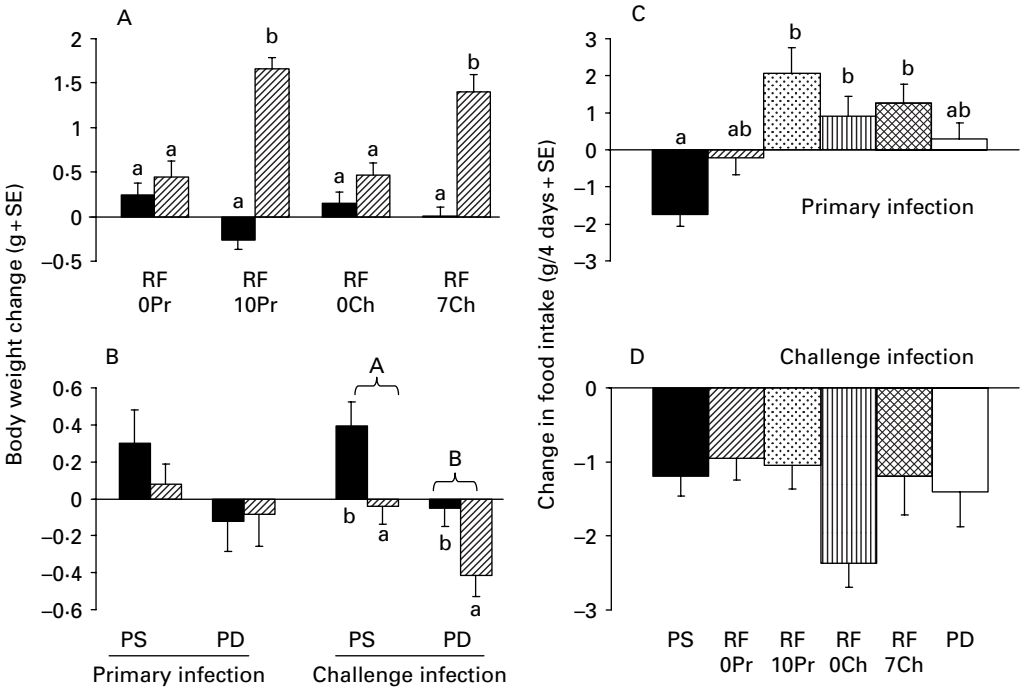

Fig. 2. Influence of infection and re-feeding on weight gain and food intake. (A) Change in body weight over the 4 days prior to re-feeding (solid bars) and the 4 days following re-feeding (cross-hatched bars) in the 4 re-feeding groups (groups defined in legend to Fig. 1). Different lower-case letters indicate differences among all groups ($P<0{\cdot}05$). Comparison of feed efficiency 4 days prior and after re-feeding showed the same pattern as body weight change. (B) Change in body weight over the 4 days prior to (solid bars) or after (cross-hatched bars) primary or challenge infection in mice fed either the protein sufficient (PS) and protein deficient (PD) diet. Diet effects ($P=0{\cdot}0009$) shown by upper-case letters; differences in weight gain before and after challenge infection shown by lower-case letters ($P=0{\cdot}001$). (C) Difference in cumulative food intake in the 4 days after primary infection compared with the 4 days prior to primary infection. Different lower-case letters indicate differences among groups ($P<0{\cdot}05$). (D) Difference in cumulative food intake in the 4 days after challenge infection compared with the 4 days prior to challenge infection.

experiment ($P=0{\cdot}049$) although no overall significant difference was detected among the 6 groups (Table 2).

Tissue damage

As general indicators of tissue damage resulting both from the infection and from the protein deficiency, sera were assayed for total ALP as well as intestinal and LBK isoenzymes. At day 10 p.c.i., total ALP of the PS mice was similar to that of all other diet groups (Fig. 3A), but was significantly higher in RF10Pr and RF0Ch than in RF7Ch and PD mice (Fig. 3A), suggesting that longer re-feeding led to higher total serum ALP. By day 28 p.c.i., total ALP was significantly higher in PD than PS mice, and total ALP was also significantly elevated in the RF0Ch mice (Fig. 3B), the group of mice where restoration of body weight had been prolonged (Fig. 1; Table 2). Significantly increased concentrations of LBK ALP and slightly higher intestinal ALP on day 28 p.c.i. underscored the elevations in total ALP seen in the PD and RF0Ch mice compared with the other re-feeding groups (Fig. 3C). We also found positive linear relationships between day 28 p.c.i. measurements of total ($P=0{\cdot}0045$), intestinal ($P=0{\cdot}0001$) and LBK ($P=0{\cdot}02$) ALP and total days of consumption of the PD diet, suggesting lingering metabolic stress to specific organs.

DISCUSSION

Our experiment was designed to determine whether the benefits of re-feeding on restoration of functional protection (measured by expulsion of adults from the GI tract) required adequate protein at a critical time during either the primary or challenge infection. We chose 4 re-feeding times, 2 during the primary infection and 2 during the challenge infection. As protein status is restored within a few days of re-feeding (Qu *et al.* 1996), our first hypothesis that mice re-fed either on the day of primary infection (RF0Pr) or on the day immediately after drug treatment (RF10Pr) (when parasite antigen would likely still be present), would have mounted a fully competent priming response to *H. bakeri* was supported by our data on egg production, worm numbers and serum IgE levels. Our earliest indicators (serum IgE on day 10 p.c.i. and egg production day 16 p.c.i.) as well as all subsequent indicators (serum IgE on day 28 p.c.i., net and *per capita* egg production and worm numbers at day 28 p.c.i.) were all

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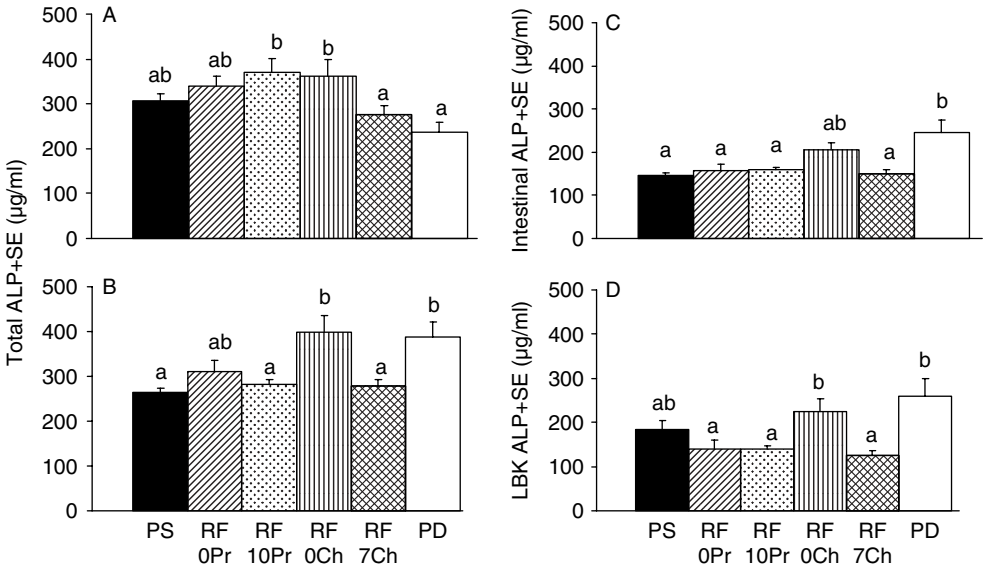

Fig. 3. Serum concentrations of total alkaline phosphatase (ALP) at day 10 (A), or day 28 p.c.i. (B) with *Heligmosomoides bakeri* and of intestinal ALP (C) and liver-bone-kidney (LBK) ALP (D) on day 28 p.c.i. (groups defined in legend to Fig. 1). Different lower-case letters indicate significant differences among groups ($P<0{\cdot}05$).

comparable to those of PS mice. Thus, protein deficiency throughout the primary infection did not reduce the ability of re-fed mice to reject a challenge infection. Although protein deficiency causes atrophy of lymphoid tissues including MLN (Deitch *et al*. 1992), interferes with germinal centre development (Bell *et al*. 1976) and homing of lymphocytes to mucosal tissues (McDermott *et al*. 1982), sufficient antigen presentation apparently occurred during the primary infection to permit an adaptive response to challenge. In fact, we have shown that parasite-specific immune effectors are present during a primary infection in PD mice (Ing *et al*. 2000), albeit at significantly lower levels that those seen in the PS controls.

We had further hypothesized that the nutritional status of mice re-fed either on the day of challenge (RF0Ch) and more particularly, those re-fed 7 days later (RF7Ch), would not permit a fully operational immune response to the challenge infection, and thus that worm survival would be prolonged as it was in PD mice. This was not supported by our data. Mice in both challenge re-feeding groups were able to clear the challenge infection with the same efficiency as mice fed the PS diet throughout the study. Based on these observations, we suggest that immunological and inflammatory responses rapidly returned to normal but at an energetic cost to the nutritionally compromised animal. In well-nourished hosts, T cells release cytokines very rapidly after challenge (Garcia *et al*. 1999; Rogers *et al*. 2000) in a primed host. Protein re-feeding is also known to rapidly restore gut structure (Qu *et al*. 1996) and non-specific inflammatory responses (Ikeda *et al*. 2001). In addition, the high proportion of quiescent CD4+ T lymphocytes found in uninfected PD mice (Woodward *et al*. 1999) and malnourished children (Najera *et al*. 2001) are presumably immediately responsive to re-feeding. Our results differ from those of Oarada *et al*. (2002) who showed that re-feeding on the day of infection effectively restored protection against fungal infection in mice, but re-feeding even one day later was less effective. It is important to note that clearance of *H. bakeri* cannot begin until the larval parasites have emerged into the intestinal lumen, an event that occurs after 7 days of challenge. This may explain why we detected no differences in worm numbers between mice re-fed on the day of challenge infection or 7 days after challenge infection.

The literature on the effect of protein deficiency on food intake is inconsistent. Some studies have reported increased food intake in mice fed a moderately low protein diet (Swick and Gribskov, 1983; White *et al*. 1994) whereas others have reported no change (Boulay *et al*. 1998; Ing *et al*. 2000) and still others found that food intake decreased (Mercer *et al*. 1994). Discrepancies among studies may be related to the differences in degree of deficiency (Ing *et al*. 2000), other dietary components (Menaker and Navia, 1973; Boulay *et al*. 1998), or genetic background or age of animals (White *et al*. 2000). None of these explanations, however, would account for why average daily intake of the PD diet prior to re-feeding exceeded average intake of the PS diet after re-feeding by 10–23%. Interestingly, however, we demonstrated that serum leptin was positively associated with food intake in these infected animals. Most studies have shown reduced serum leptin in PEM (Haluzik *et al*. 1999; Soliman *et al*. 2000; Palacio *et al*. 2002) with the exception of Stapleton *et al*. (2003). Our data in infected mice, if anything,

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suggest higher leptin in PD mice than in PS mice. Elevated leptin has been reported during bacterial infection (Arnalich *et al*. 1999), during inflammation (Barbier *et al*. 1998; Matarese *et al*. 2005) and during the early stage of *Nippostrongylus brasiliensis* infection (Roberts *et al*. 1999). Therefore, it is possible that adaptation to the PD diet together with prolonged infection-induced inflammation in PD mice led to increased leptin concentrations and higher food intakes in infected PD mice.

Weight loss is not normally reported in studies on *H. bakeri* (Slater and Keymer, 1986; Shi *et al*. 1994; Kristan and Hammond, 2000), perhaps because researchers focus on weight at the end of the study, rather than immediately after larval exposure. Our data clearly show a temporary cessation of weight gain at the time of larval challenge in PS mice, and actual weight loss in PD mice. We suggest that this could result from 3 factors. First, food intake was reduced in all diet groups during the 4 days after larval challenge, compared with the 4 days preceding challenge infection. Second, protein deficiency alone increases intestinal paracellular permeability (Rodriguez, 1996), and within 24 h of infection, the penetration of L_3 larvae into the mucosa causes haemorrhaging and net water loss (Sukhdeo and Mettrick, 1984; Kamal *et al*. 2002). Thus, the water loss resulting both from active penetration of L_3 larvae and leakage due to protein deficiency may explain the weight loss immediately following infection. Finally, reduced weight gain may also be a consequence of the allocation of resources to the rapid immunological response triggered by larval challenge. IL-4 production is extremely high within 8 h of challenge, IL-13 first peaks at day 3 p.c.i. and concentrations of both are substantially higher than during a primary infection (Finkelman *et al*. 2000). Mucosal mast cell activation and degranulation are also induced within 3 days of larval challenge (Ing *et al*. 2000), even in PD mice. Therefore, we posit that the reduced weight gain observed over the 4 days following challenge infection reflects water loss and anorexia, together with preferential allocation of resources to host responses rather than to growth.

Nutritional status was restored rapidly in the RF7Ch group but much more slowly in the RF0Ch group where indicators of tissue damage (both total and intestinal ALP) were higher even on day 28 p.c.i. than those of PS mice. Our data thus indicate that protein re-feeding that begins as late as 7 days after challenge infection is able to restore functional protection measured by egg production approximately 1 week later, and by worm numbers present on day 28 p.c.i. Catch-up growth following re-feeding of protein-deficient rats normally occurs within 3 days (Qu *et al*. 1996), an observation consistent with our data for the RF10Ch and RF7Ch groups of mice. However, larval challenge dramatically delayed the catch-up growth of mice re-fed on the same day as larval challenge (RF0Ch mice). Compared with a primary infection, challenge infection induces more prominent changes both in the parasite-specific immune responses and in the architecture and physiology of the gut. At the local level, IL-4 expression by MLN cells remains elevated for 2 weeks (Ing *et al*. 2000), mucosal mastocytosis and eosinophilia is elevated as early as day 3 p.c.i. and persists at least for 28 days (Ing *et al*. 2000; Shea-Donohue *et al*. 2001), and mucosal mast cell protease-1 (mMCP-1), a peripheral marker of mast cell degranulation, increases to a peak on day 14 p.c.i. (Ing *et al*. 2000). Intestinal glands are enlarged and an intense cellular reaction with neutropolymorphonuclear cells, plasma cells, and eosinophils is evident in the mucosal epithelium, along with an increase in number and size of goblet cells and in the amount of mucus present in the lumen of the intestine (Cywinska *et al*. 2004). In addition, elevated mucosal permeability of jejunal epithelial cells along with reduced Na^+-linked glucose absorption and increased Cl-secretory response to prostaglandin E2 occur on day 10 p.c.i. (Shea-Donohue *et al*. 2001). Thus, it is likely that the demands on host protein imposed by this cascade of responses delays the ability of the host to divert the additional dietary protein into body growth, and that this explains the very much longer catch-up growth period observed in the mice re-fed on the same day as larval challenge.

Concentrations of ALP have been reported in a variety of studies examining protein deficiency and re-feeding as well as helminth infection in rodents, rabbits, monkeys and humans, but no consistent pattern has emerged. Some studies report that ALP is reduced by protein deficiency (Pond *et al*. 1992; Rana *et al*. 1995) whereas others indicate the opposite (Rana *et al*. 2003; Sidhu *et al*. 2005). Similarly, ALP is reduced in *Trichostrongylus colubriformis* (Stankiewicz *et al*. 1996) and *Clonorchis sinensis* (Hong *et al*. 1994) infection but elevated in *Schistosoma mansoni* (Mansour *et al*. 1982) and *H. bakeri* (Boulay *et al*. 1998) infection. These apparently conflicting results may stem from differences in the species, the infection and/or the severity of deficiency. In the classic model, protein deficiency in humans induces a reduction in serum ALP that progresses with duration and severity of the deficiency. In mild protein deficiency in children, ALP rapidly returns to control levels, but in more severe deficiency, ALP remains depressed for a period after re-feeding, then rebounds and may increase to levels above that of normal children (Schwartz, 1956). This sequence of events in humans is consistent with our mouse data. Those mice re-fed early experienced only limited dietary protein deprivation, and early re-feeding would have resulted in a rapid return to control levels by day 10 p.c.i. In contrast, both our PD mice and those re-fed later were protein deficient for a longer period of time and would have

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experienced a more severe deficiency that might explain the low day 10 ALP levels in our PD and RF7Ch mice. The relatively higher values in RF10Pr and RF0Ch would be consistent with a rebound ALP that is somewhat elevated following more prolonged protein deficiency. By day 28 p.c.i., ALP levels were restored to normal in all groups except PD mice and the RF0Ch group. It is easy to attribute the elevated ALP in the PD group of mice to continuing damage induced by PD (Li *et al*. 1989) and infection (Boulay *et al*. 1998). What is more surprising is the elevated ALP on day 28 p.c.i. in the RF0Ch group, the only group of mice where re-feeding began on the same day as larval challenge, and where restoration of body weight took 3 times as long as in all other re-feeding groups. Apparently, larval challenge concurrent with re-feeding caused a substantially higher physiological stress in these animals. This is supported by the elevated LBK ALP known to be associated with hepatic involvement in resolution of tissue damage due to infection.

Based on our results, several key points emerge. We have confirmed that protein deficiency impairs the ability of mice to reject a challenge infection with *H. bakeri*, and have shown for the first time that re-feeding a protein sufficient diet during either a primary or challenge infection restores host clearance of this GI nematode. We have also shown that catch-up growth following re-feeding allows mice to attain normal body weights within 4 days, unless they are simultaneously exposed to *H. polgyrus* L_3. Based on these observations together with our previous results, we suggest that even a 3% diet provides adequate protein for priming of a parasite-specific immune response. So long as a protein-sufficient diet is available for at least a week during the challenge infection, mice are able to mount the appropriate immunological and/or inflammatory responses required to reject the parasite. We suggest that the protein deficiency–protein re-feeding paradigm may be extremely useful in defining the thresholds of various effectors needed for protection.

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