The effect of cysteine and glutamine supplementation on sheep infected with *Trichostrongylus colubriformis*.

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ABSTRACT

This indoor study investigated the effect of direct daily abomasal supplementation of both cysteine (cys; 1g) and glutamine (gln; 5g) on a 12-week subclinical *Trichostronglylus colubriformis* trickle infection in lambs. Parasite infection increased the total number of leucocytes and eosinophils in peripheral circulation, nitrogen excreted in faeces and urine ($P \leq 0.01$) and plasma total protein concentration ($P < 0.05$). Infection also reduced liveweight gain and plasma albumin concentration (both $P < 0.001$), but did not alter feed intake. Supplementation increased nitrogen retention ($P < 0.05$), tended to increase phe ($P = 0.056$) and cys ($P = 0.097$) flux, reduced the number of circulating eosinophils ($P < 0.001$) and changed the pattern of faecal egg counts ($P \leq 0.05$), with no effect on final nematode counts. This experiment showed supplementation with two non-essential amino acids alone can elicit changes in the response of sheep to internal parasite infection.

Keywords: amino acids; internal parasites; sheep; protein flux; eosinophilia.

INTRODUCTION

Reducing the impact of internal parasites on ruminant livestock can be achieved by feeding high protein diets and/or rumen bypass protein and/or forages containing condensed tannins (for example, Bown *et al.*, 1991; Donaldson *et al.*, 1997; Hoskin *et al.*, 2000; Houdijk *et al.*, 2001). However, supplementation with specific amino acids may elicit similar responses and recent knowledge indicates that responses by animal defence mechanisms to infection involve the amino acids cysteine (cys) and glutamine (gln) (Malmezat *et al.*, 1998; Lobley *et al.*, 2001).

Demand on muscle cysteine stores by the immune system is thought to arise from synthesis of acute-phase proteins, leucocytes plus antioxidants glutathione, and taurine (Breuille and Obled, 2000; Malmezat *et al.*, 1998; 2000). Glutamine is an obligate fuel and precursor for *de novo* protein and nucleic acid synthesis (Gate *et al.*, 1997). A high demand for glutamine comes from proliferative immune cells such as lymphocytes and the cells of the gastrointestinal (GI) tract. Glutamine supplementation has been shown to assist in the recovery of surgically-damaged gut tissue and hence may have a role in enhancing repair of parasite-damaged intestinal mucosa (see Lobley *et al.*, 2001).

The purpose of this study was to investigate the effect of abomasal supplementation of two non-essential amino acids, cysteine and glutamine, on *Trichostronglylus colubriformis* infection in lambs.

MATERIALS AND METHODS

Animals and treatments

Four castrate twins and 6 entire male Suffolk X single lambs aged 6-7 months, reared parasite naïve indoors, with mean live weight ± SD (45.1kg ±2.48) were surgically prepared in March 1999 with permanently indwelling abomasal polyvinyl chloride cannulas and allowed to recover for 4 weeks. Lambs were paired according to twinning or a combination of sex, age and live weight and randomly allocated to 2 treatments: control (CON), receiving a daily infusion of 10ml water, or supplemented (SUP), receiving 1g cysteine and 5g glutamine (pure amino acid, Ajinomoto) dissolved in 10ml water syringed into the abomasal cannula in a single shot during non-measurement periods and continuously infused by peristaltic pump during measurement periods.

Animals were pair-fed dried grass pellets (10MJ estimated metabolisable energy/ kgDM; 26 g N/kg DM) at a maximum intake of 2.0 x energy maintenance (based on 400kJ/kg body weight$^{0.75}$) prior to parasite infection and at 2.5 x energy maintenance thereafter. Animals were individually housed in floor pens during non-measurement periods and fed twice daily, and in metabolism cages during adjustment (5d) and measurement (7d) periods whilst being fed equal portions hourly by automatic feeders, with lighting set to 8h dark, 16h light.

Infusion, blood sampling and nitrogen balance

Prior to infection and at weeks 4, 8 and 12 of the infection, a temporary polyvinyl catheter was inserted into each jugular vein (filled with dextrose plus sodium citrate, see Hoskin *et al.*, 2001), one for tracer infusion and the other for blood sampling. Faecal and urine collection harnesses were fitted for nitrogen balance measurements during each measurement period. On the second to penultimate day of each measurement period, 2 x 10ml blood samples were taken for haematology and plasma biochemistry measurements and for background (initially natural abundance) enrichment samples. On the final day of the measurement period a 9h continuous infusion (4g/h) of [1-^{13}C]-cysteine (100mg) and [$^{2}H_{5}$]-phenylalanine (300mg) dissolved in 50ml sterile 0.15M-NaCl was initiated. Spot blood samples (5ml) were taken approximately every 30 minutes from 5h after the start of infusion.

Parasite infection

Pure, sheep-origin, infective stage *T.colubriformis* larvae (Moredun Research Institute) were administered

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by mouth 5 days per week (4,000 larvae per dose) for 12 weeks to achieve a subclinical infection. Sheep were weighed and faeces samples taken for faecal egg counts (using a modified McMaster technique with saturated NaCl solution) weekly. Faecal egg counts were zero prior to the start of the experiment. Following the 12-week measurement period, animals were euthanased with sodium pentabarbitone and the small intestine removed for nematode counts on 10% of the small intestinal digesta washings/contents and mucosal saline digest by the parasitology laboratory, Moredun Research Institute.

Laboratory analyses

Plasma samples were deproteinised with sulfosalicylic acid (final concentration 70g/l plasma), desalted with Dowex-50 H^+ and the free amino acids eluted with 2 M-NH_4OH. Plasma free [$^2H_5$]-phenylalanine and [1-^{13}C]cysteine enrichments were determined using gas chromatograph mass spectrometry following the procedures of Connell *et al.* (1997), with inclusion of dithithreitol in the extraction stage to aid release of protein-occluded cysteine. Haematology analyses were performed on fresh blood samples at the Scottish Agricultural College Veterinary Science Division, Aberdeen. Plasma albumin, total protein and urea concentrations were determined from frozen plasma using a Kone Clinical Analyser (Kone Ltd, Espoo, Finland).

Calculations

Daily protein flux (PrF; g/d) for cysteine and phenylalanine (phe) were calculated by a conversion of the irreversible loss rate (IRL = (0.99/ enrichment of free cys or phe in plasma – 1) x infusion rate of labelled amino acid tracer; mmol/h; Savary *et al.* (2001)).

$PrF = IRL \times 24 \times MW_{cys\ or\ phe} / protein\ content_{cys\ or\ phe}$)

MW is the molecular weight of amino acid (121 cys / 165 phe) and protein content is the weight of amino acid per 100g mixed ovine protein deposited during growth (1.4 cys / 3.6 phe; from MacRae *et al.*, 1993).

Statistics

All statistical analyses were carried out with Genstat 5.4.1 (Lawes Educational Trust, Rothamsted, Herts, UK). P values <0.05 were considered significant and P values <0.10 to represent trends. Data were analysed by ANOVA, with animals and pairs initially treated as blocks and supplementation x week as treatment. The majority of data gave showed no pair effect and this was then excluded to increase the degrees of freedom (except haematology, apparent nitrogen retention and nematode counts). In addition, initial testing showed no improvement when pre-infection measurements were included as a covariate for post-infection measurement periods.

RESULTS

There was no effect of internal parasitism or amino acid supplementation on feed intake or dry matter digestibility (data not shown). Treatment did not affect weekly FEC, but the mean peak FEC value was greater

TABLE 1: Numbers of *Trichostrongylus colubriformis* recovered from the small intestine at slaughter, the peak faecal egg count (FEC) and time taken to reach the peak FEC during 12 weeks of trickle infection in 6-month-old sheep receiving abomasal cysteine and glutamine supplementation (Supplemented), or unsupplemented (Control).

	Control	Supplemented	SEM	P
Total nematodes (No)	39600	32980	6933.5	NS
Peak FEC (eggs/g faeces)	2372	1561	154.4	0.021
Time Peak FEC reached (week)	7.2	5.0	0.57	0.051

TABLE 2: Liveweight gain and nitrogen (N) excretion in faeces and urine prior to, and at, 4-weekly intervals during 12 weeks of trickle *Trichostrongylus colubriformis* infection in 6-month-old sheep.

	*Pre-infection**	Week 4	Week 8	Week 12	SEM	P week
Liveweight gain (g/d)	*150.7*	200.0^{a}	144.7^{a}	30.4^{b}	16.10	<0.001
Faecal N (gN/d)	*18.42*	22.18^{a}	25.96^{b}	24.56^{ab}	1.257	<0.001
Urinary N (gN/d)	*17.46*	21.66^{a}	23.64^{ab}	25.29^{b}	1.238	0.010

*ANOVA comparisons are between weeks 4, 8 & 12 only, because pre-infection feeding level (2.0x EM) differed to post-infection (2.5x EM) which directly influences N excretion and liveweight gain. ^{ab}Different letters indicate significant differences between weeks (P<0.05).

TABLE 3: Plasma total protein, albumin and urea concentrations and haematology measurements of 6-month-old sheep prior to, and at, 4-weekly intervals during 12 weeks of trickle *Trichostrongylus colubriformis* infection.

	Pre-infection	Week 4	Week 8	Week 12	SEM	P week
Plasma Concentrations						
Total protein (g/l)	66.27^{ab}	62.39^{a}	68.82^{b}	64.80^{ab}	1.995	0.025
Albumin (g/l)	30.35^{a}	29.90^{a}	28.80^{ab}	27.15^{b}	0.693	<0.001
Urea (mmol/l)	5.85^{ab}	5.82^{a}	6.85^{ab}	7.09^{b}	0.429	<0.001
Haematology						
Erythrocytes (x10^{12}/l	9.49	9.64	9.79	9.53	0.507	NS
Packed cell volume (%)	31.7	30.8	31.9	31.5	1.49	NS
Total leucocytes (x10^{9}/l)	7.68^{a}	8.38^{ab}	9.75^{ab}	10.30^{b}	0.921	0.001
Differential lymphocyte counts (x10^{9}/l)	5.70	6.10	6.77	7.23	0.734	0.065
Differential monocyte counts (x10^{9}/l)	0.157^{a}	0.131^{ab}	0.000^{b}	0.083^{ab}	0.0516	0.028

ANOVA comparisons presented are between all measurement periods. ^{ab}Different letters indicate significant differences between weeks (P<0.05). No infection*supplementation interactions were found for any of this data.

TABLE 4: Number of circulating eosinophils and apparent nitrogen retention in 6-month-old sheep, prior to, and at, 4-weekly intervals during 12 weeks of trickle infection with *Trichostrongylus colubriformis* and receiving abomasal cysteine and glutamine supplementation (Supplemented), or unsupplemented (Control).

	Control		Supplemented
Differential Eosinophil Counts ($x10^9$/l)*			
0 (*pre-infection*)		*0.12*	
4	0.10		0.12
8	0.84[a]		0.24[b]
12	1.5[a]		0.82[b]
SEM		0.20	
P Treatment		0.022	
Apparent Nitrogen Retention (g N/d)**			
0 (*pre-infection*)		*6.1*	
4	8.1		9.4
8	5.8[a]		9.1[b]
12	5.8		7.8
SEM		0.86	
P Treatment		0.025	

[ab]Different letters indicate significant differences between treatments within weeks (P<0.05). *A significant effect of week of infection on peripheral eosinophil counts was found (P<0.001). **There was no significant effect of week of infection on nitrogen retention (P>0.10)*. No infection*supplementation interactions were found for any of this data.

TABLE 5: Whole body protein flux calculated using cysteine and phenylalanine, prior to, and at, 4- weekly intervals during 12 weeks of trickle *Trichostrongylus colubriformis* infection in 6-month-old sheep receiving abomasal cysteine and glutamine supplementation (Supplemented), or unsupplemented (Control).

	Control		Supplemented
Whole body protein flux (g/d based on cysteine kinetics)			
0 (*pre-infection*)		*644*	
4	703		775
8	720		808
12	686		789
SEM		36.7	
P Treatment		0.097	
Whole body protein flux (g/d based on phenylalanine kinetics)			
0 (*pre-infection*)		*552*	
4	6558		717
8	656[a]		769[b]
12	702		724
SEM		28.3	
P Treatment		0.056	

Cysteine

ANOVA comparisons are between weeks 4, 8 & 12 only, because pre-infection feeding level (2.0x EM) differed to post-infection (2.5x EM), which directly influences whole body protein flux. [ab]Different letters indicate a significant difference between treatments at week 8 for phenylalanine flux (P<0.05). There were no significant week of infection*supplementation interactions.

and the time when peak FEC was reached was earlier in the SUP vs CON groups (P≤0.05; Table 1). For liveweight gain, urinary and faecal nitrogen losses (Table 2) there was an effect of week of infection (P≤0.01), but not treatment response In contrast, the SUP treatment did improve net nitrogen retention, particularly at week 8 (P<0.05 Table 4).

Plasma concentrations of urea, albumin (Table 3) and phosphorus (data not shown) were not influenced by amino acid supplementation, but there was a trend for increased plasma total protein concentration in the CON, group (P=0.051), most noticeably at week 8 (CON 72.78 vs SUP 64.86 g/l; P<0.05). Parasite infection caused a reduction in plasma albumin concentration (P<0.001) and an increase in plasma total protein concentration ((P<0.05; by difference due to increasing globulin concentration (data not shown), again particularly at week 8.

Parasite infection increased the number of leucocytes in the peripheral circulation (P<0.01), but there was no treatment effect. There was no change with infection or treatment in the number of circulating neutrophils (data not shown), but, despite no effect of treatment, lymphocyte number tended to increase (P=0.065) and monocyte counts decreased (P<0.05) as infection progressed (Table 3). Numbers of circulating eosinophils (Table 4) were lower for SUP than CON in weeks 8 and 12 (both P<0.05), with an overall week effect (P<0.001).

The IRL's (mmol /h) for cysteine and phenylalanine, from which the protein fluxes presented in Table 5 were calculated, are for cysteine (SEM 0.182; P treatment 0.097): pre-infection mean 3.10, week 4, 3.41(CON) vs 3.73(SUP), week 8, 3.49(CON) vs 3.88(SUP), week 12, 3.28(CON) vs 3.76(SUP). Phenylalanine IRL's (SEM 0.331; P treatment 0.056) were: pre-infection mean 5.02, week 4, 5.95(CON) vs 6.52(SUP), week 8, 5.96(CON) vs 6.99(SUP), week 12, 6.39(CON) vs 6.58(SUP) with the greatest response at week 8 (P<0.05). The ANOVA for whole body protein flux data exclude the pre-infection period data. Inclusion did produce significant effects (P<0.05), but these could not be resolved between responses to intake or parasite infection. Overall, the data for cysteine and phenylalanine suggest an increased flux for SUP vs CON (cys P=0.097; phe P=0.056), with again the greatest response at week 8 (P<0.05).

DISCUSSION

Currently there is increased interest in sustainable, non-chemical, nutrition-based methods to either control internal parasites or increase the resilience and/or resistance (Coop & Kyriazakis, 1999) of the host animal. Yet, despite recent research into the effect of protein supplementation on parasitic and other immunological responses in sheep, the requirements for specific amino acids remains poorly understood. The lack of knowledge is even greater where the non-essential amino acids are concerned, despite indications from human research that some of these have the potential to become 'conditionally essential' in times of high demand such as an immune challenge or following trauma/surgery (Lacey & Wilmore, 1990).

Cysteine and glutamine supplementation reduced peripheral eosinophilia and increased apparent nitrogen retention of parasitised sheep. However, despite the improvement in apparent nitrogen retention, live weight gain remained inhibited during the latter period of infection. The lack of a growth response to supplementation may be due to a) an insufficient dose being applied; b) the need for other co-limiting amino acids to be given; c) neither cysteine nor glutamine being limiting under the challenge conditions employed, or d) that the potential for growth expression in the sheep declined during this experiment. Given the age and starting live weight of the sheep used in the current trial

it is likely that as they were approaching mature age and live weight in the latter stages of the experiment, their potential for growth was declining. However, the absence of an uninfected control group in the trial design does not allow this to be determined conclusively and it is suggested that future work of this type involve younger animals and/or an uninfected control group. The selection of the dosages'was based on knowledge of absorption for the diet used (supply), data for whole body flux (loss), and glutathione synthesis rates (estimated as a primary demand for cysteine) from previous trials and consideration of the dangers of over-supplementation with sulphur amino acids. Recently, Miller *et al*. (2000) reported responses to larger dosages of cysteine alone (2g/d), which increased the plasma concentration of cysteine by 65%, but plasma concentration of cysteine was not measured in the current study.

Supplementation increased the cysteine flux by approximately 1g/d, equivalent to the dose. This may reflect either a) complete absorption and transfer to the peripheral circulation; and/or b) an increase in endogenous flux. However, complete quantitative transfer from the gut lumen to the periphery is unlikely (McNabb *et al*., 1993; Berthiaume *et al*., 2001). Furthermore, there was an increase (14%) in whole body flux, based on non-supplemented phenylalanine kinetics. Probably, therefore, the cysteine flux changes reflect, in part, increases in either the rate of removal (protein synthesis or oxidation) or appearance (from protein breakdown). Such responses may involve tissue-specific changes in amino acid partitioning, including at the GI tract (Yu *et al.*, 2000; Jones & Symons, 1982; Symons & Jones, 1975).

Total protein supplementation can increase resilience (hosts ability to maintain reasonable production in the face of parasite challenge), rather than resistance (host reducing establishment, growth rate, fecundity and or persistence of parasite population) to internal parasites (Coop & Kyriazakis, 1999). Such responses probably involve requirements for specific amino acids rather than protein *per se*. Furthermore, the needs for specific amino acids will probably change throughout the progress of infection. The effectiveness of single amino acid supplementation has been shown for methionine-supplemented lambs fed a low protein concentrate ration and infected with *T.colubriformis*, in which both wool growth and rate of liveweight gain were improved compared with un-supplemented lambs on the same ration, despite no effect on FEC (Coop *et al.*, 1997). The current study reinforces this concept , with increased nitrogen retention (but not enough to reflect changes in liveweight gain), but without effects on either apparent parasite 'establishment' or weekly mean FEC.

The reduced response of peripheral eosinophilia to amino acid supplementation in the current study is intriguing and contrasts with the increased eosinophilia observed for cysteine-supplemented sheep in the study of Miller *et al*. (2000). A reduced response in peripheral eosinophilia under parasite challenge is a possible indication of increased resistance to infection, but is usually accompanied by lowered FEC, which was not the observed in the current study.

Further interpretation of FEC data revealed a lower, and earlier, peak in FEC in the supplemented group, but the biological significance of this is unclear. However, when this information is, taken together with the strong response in peripheral eosinophil number and a 17% (non-significant) reduction in nematode counts in this group, there is indication of increased resistance. This may have become clearer by either extending the trial beyond 12 weeks or by increasing the amount of supplement. That cysteine supplementation of previously naturally parasitised sheep that were treated and re-infected artificially with both *T.colubriformis* and *H.contortus* by Miller *et al.* (2000) produced a two-fold (but non-significant) decrease in FEC and also increased wool growth by 6-21% (again, non-significant) , indicates that appropriate amino acid supplementation may be able to alter resistance, as well as resilience.

In summary, although supplementation of parasitised sheep with cysteine and glutamine produced lesser responses than would be predicted from a general protein supplement, the need to identify exact metabolic requirements during such challenges remains. Such selective targeting would lower the costs and pollution associated with over-provision of protein components and yet still assist the natural defence mechanisms of the animal and reduce reliance on chemical and pharmaceutical controls.

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