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Arginine-Derived Nitric Oxide Reduces Fecal Oocyst Shedding in Nude Mice Infected with *Cryptosporidium parvum*

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Dietary L-arginine (4%) significantly reduced fecal oocyst shedding in athymic nude mice chronically infected with *Cryptosporidium parvum*. This effect appeared to be due to an increase in host nitric oxide (NO) production as it was not observed in arginine-supplemented animals administered the NO synthase inhibitor, *N*-nitro-L-arginine methyl ester. *N*-Nitro-L-arginine methyl ester alone significantly increased fecal oocyst shedding in chronically infected animals. In in vitro assays, oocyst excystation and sporozoite viability were significantly reduced by the NO donors sodium nitroprusside and *S*-nitroso-L-acetyl penicillamine in a concentration-dependent manner. These data suggest that arginine-derived NO may reduce the parasite load in experimental cryptosporidiosis.

Cryptosporidium parvum is an intracellular protozoan parasite that establishes itself in a parasitophorous vacuole just within the brush border of host small intestinal enterocytes (6). The infection is self limiting, except in immunodeficient hosts, in which it may cause protracted and untreatable diarrhea. As a result, chronic cryptosporidiosis is responsible for considerable morbidity and some mortality in AIDS patients (16). Athymic nude mice (10, 13, 21) and SCID mice (17) have been used to establish chronic *C. parvum* infections similar to those seen in AIDS patients, and while such models have shortcomings in terms of being able to mimic the diarrhea seen in human infections, they are useful for studies of host-parasite interactions and to test putative chemotherapeutic agents.

The present study was undertaken to determine whether, and to what extent, nitric oxide (NO) played a role in controlling the parasite load in experimental cryptosporidiosis. Adult male athymic nude mice were individually housed in filter top cages after being infected with *C. parvum*, originally of calf origin, as described elsewhere (13). Five fecal pellets were collected daily in 2 ml of neutral buffered formalin, and the oocysts suspended in a 10-μl sample of fecal homogenate were counted after being visualized with a commercial direct immunofluorescence assay that employs a fluorescein isothiocyanate-labeled monoclonal antibody directed against *Cryptosporidium* oocyst wall antigens (Meridian Diagnostics, Inc., Cincinnati, Ohio). An oocyst shedding score (0, no oocysts; 1, 1 to 10 oocysts; 2, 11 to 50 oocysts; 3, 51 to 100 oocysts; 4, >100 oocysts) that correlated well with the number of both infected ileal villus enterocytes and crypt cells and correlated negatively with the ileal villus-to-crypt length ratio was developed (13). Thus, the shed fecal oocyst score could be used as an index of parasite load.

Animals that had been infected with 10^5 oocysts more than 3 weeks earlier were fed a control diet ad libitum (powdered Purina Lab Chow). Control animals were maintained on this diet, while other animals were fed this diet to which had been added 4% L-arginine or 4% L-citrulline. All diets were autoclaved at 160°C for 20 min, while injected agents were dissolved in phosphate-buffered saline (PBS) and filter sterilized.

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Animals fed the control diet showed a gradual increase in fecal oocyst score over a 2-week period while animals on the arginine-supplemented diet showed a decrease in oocyst shedding. Figure 1 illustrates the effects of feeding animals the various diets for 14 days on the change in shed oocyst score, i.e., the difference in score between the value on a given day and that on the day prior to when the test diet was first administered (day 0). Each diet group contained eight animals with day 0 oocyst scores ranging from 1 to 4. The range of mean day 0 scores was 2.75 to 3.13. Animals on the control diet showed a significant increase ($P < 0.01$) in shed oocyst score during the test period as determined by a one-way analysis of variance (ANOVA). Changes in oocyst shedding scores were significantly less ($P < 0.01$) in the arginine-supplemented diet group compared with the control group as determined by a two-way ANOVA, even after the animals had been returned to the control diet ($P < 0.05$ on day 19, Tukey's protected t test).

Arginine is known to protect against a variety of infectious agents (9), and this protection is generally ascribed to the generation of NO by NO synthase. L-Arginine is converted to NO and citrulline by this enzyme (18). To ensure that citrulline was not causing the observed reduction in fecal oocyst shedding, animals were fed chow supplemented with 4% L-citrulline for 14 days. Figure 1 illustrates the lack of any antiparasitic effect of the citrulline-supplemented diet. No antiparasitic effect was seen in animals fed the 4% arginine-supplemented diet for 14 days when these animals were also administered the NO synthase inhibitor, *N*-nitro-L-arginine methyl ester (L-NAME), at 80 mg/kg of body weight subcutaneously (s.c.) twice daily. This dose of L-NAME was without any detectable toxicity when given for 14 days. The data illustrated in Fig. 1 suggest that the antiparasitic effect of dietary arginine was related to host NO metabolism.

In another experiment, animals were infected by the oral administration of 10^5 *Cryptosporidium* oocysts immediately prior to being placed on the control diet or the 4% arginine-supplemented diet. Figure 2a illustrates the shed oocyst scores of these animals. The arginine-supplemented diet group had a significantly lower ($P < 0.01$) fecal oocyst score as determined by two-way ANOVA of the data on days 7 to 19. Post hoc Tukey's protected t tests indicated that the difference between the two groups was no longer statistically significant by day 19. However, when the diets of the two groups of animals were

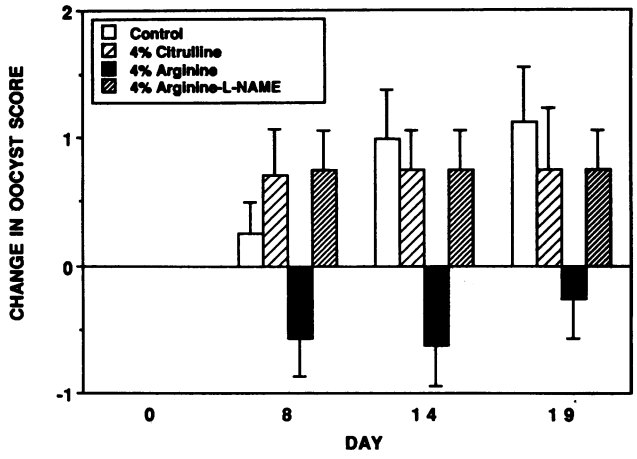

FIG. 1. Changes in fecal oocyst scores from the value on day 0. Chronically infected animals were maintained on control chow or on this chow supplemented with L-citrulline or L-arginine on days 1 to 14 before being returned to the control diet. In a second group given the L-arginine-supplemented diet, the NO synthase inhibitor, L-NAME, was administered s.c. (80 mg/kg) twice daily on days 1 to 14.

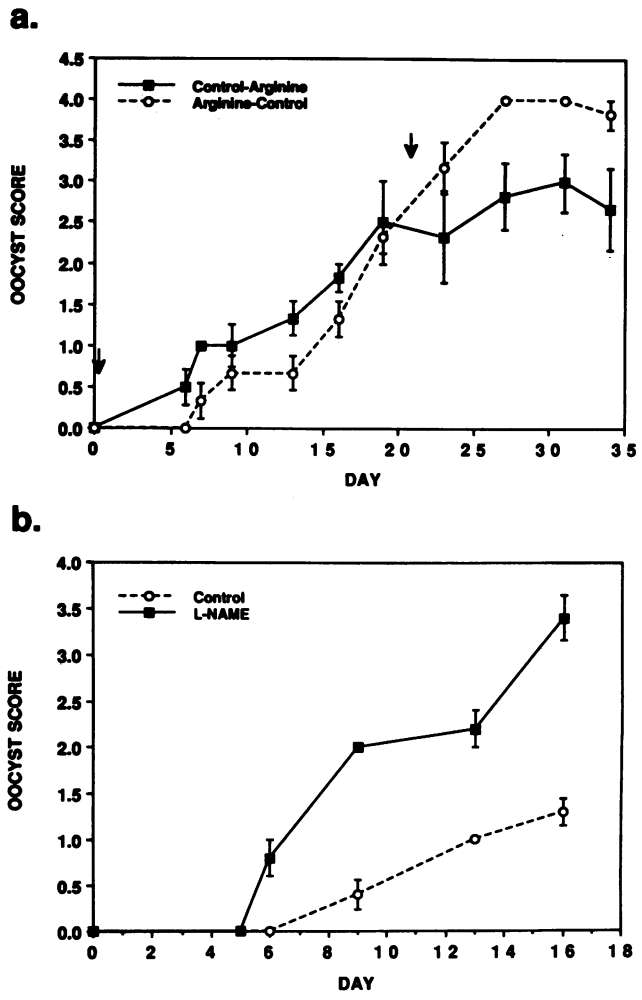

FIG. 2. (a) Effects of a 4% L-arginine-supplemented diet on shed fecal oocyst scores following oral inoculation with 10^5 *C. parvum* oocysts. At the first arrow (day 1), one group of animals was fed the control diet (open circles) and a second group received the 4% L-arginine-supplemented diet (closed circles). At the second arrow (day 21), the diets of the two groups were reversed. (b) Effects of the NO synthase inhibitor, L-NAME (80 mg/kg s.c. twice daily), given on days 5 to 9 on the shed fecal oocyst score of control diet-fed animals following the administration of 10^3 oocysts per os.

reversed on day 21, the arginine-supplemented animals again exhibited significantly lower shed oocyst scores as determined by two-way ANOVA of the data on days 23 to 34.

Two groups of animals were infected with 10^3 *Cryptosporidium* oocysts, and 5 days later, one group began a 4-day course of L-NAME (80 mg/kg s.c. twice daily). The lower oocyst inoculum number was used in this experiment so that the control animals would have a more gradual increase in oocyst shedding with time and any increase in shedding induced by the NO synthase inhibitor would be readily detected. Figure 2b illustrates that L-NAME did increase the oocyst shedding score. This increase was significant ($P < 0.01$) as determined by two-way ANOVA. The difference between the two groups persisted after discontinuing the L-NAME administration. The parasitosis continued to increase in both groups during the observation period. In the case of the L-NAME group, the elevated oocyst score suggested that the 4-day course of inhibitor boosted the infection, while the establishment of infection in the control group proceeded more slowly because of the retarding effect of some ongoing host NO-mediated process.

The NO donor, *S*-nitroso-L-acetyl penicillamine (SNAP), administered (22 mg/kg s.c. twice daily) for 7 days (peak body fluid concentration, 0.1 mM) to chronically infected animals, had no effect on the oocyst shedding score of a group of chronically infected animals (data not shown). In general, tolerance is rapidly induced with NO donors and exogenous NO inhibits endogenous synthesis, although tolerance is believed to be less of a problem with SNAP than with many of the other NO donors (20). Thus, between drug tolerance development and inhibition of host NO synthesis it is perhaps not surprising that we were unable to demonstrate an in vivo effect of an exogenous NO donor when it was injected twice daily.

NO donors were tested for direct antiparasitic effects under conditions in which drug tolerance and suppression of endogenous host NO synthase were not factors in the outcome. SNAP and sodium nitroprusside were used in an in vitro oocyst excystation system. Oocysts were stimulated to excyst by the following method (2a). Isolated oocysts were incubated at 37°C for 45 min in a solution of 0.1 M sodium acetate–0.025 M sodium chloride (pH 5.6), washed in PBS, and then incubated for an additional 45 min in 0.75% synthetic sodium taurocholate in PBS. The resulting excystation was measured by counting empty and full oocysts in glutaraldehyde-fixed samples by phase-contrast microscopy. In some oocyst samples, NO donors were included in all incubation solutions. Both NO donors significantly inhibited oocyst shedding in a dose-dependent manner as assessed by one-way ANOVA of repeated measures (Table 1).

Sodium nitroprusside and SNAP were also tested in a sporozoite viability assay. *Cryptosporidium* oocysts were excysted as described above and incubated for 30 min at 37°C in PBS or PBS containing the NO donor. Viability was assessed by the fluorescein diacetate-propidium iodide vital staining technique as adapted to *Cryptosporidium* sporozoites (3). Table 1 shows the viability of freshly excysted sporozoites after incubation in PBS (control) or PBS containing sodium nitro-

TABLE 1. Effect of NO donors on the percentage of *C. parvum* oocysts excysting following incubation in an excysting medium and on the viability of freshly excysted sporozoites

Characteristic and NO donor	% of excysted oocysts or viable sporozoites (mean ± SEM) at NO donor concn (mM)				
	0	0.001	0.01	0.1	1.0
Excystation					
Sodium nitroprusside	88.1 ± 1.6	81.6 ± 3.4	73.0 ± 2.7[a]	70.5 ± 2.2[a]	63.1 ± 2.5[a]
SNAP	88.1 ± 1.6	79.4 ± 1.0[b]	75.1 ± 2.8[a]	72.4 ± 2.0[a]	67.4 ± 3.1[a]
Viability					
Sodium nitroprusside	97.6 ± 1.2	95.8 ± 2.4	81.8 ± 5.2[b]	67.9 ± 3.2[a]	69.1 ± 5.0[a]
SNAP	90.8 ± 2.1	91.8 ± 4.4	82.8 ± 1.5[a]	70.6 ± 1.5[a]	42.0 ± 5.3[a]

[a] Significantly different from control: $P < 0.01$.
[b] Significantly different from control: $P < 0.05$.

prusside or SNAP. The NO donors significantly reduced viability as determined by one-way ANOVA of repeated measures.

The present study suggests that dietary arginine reduced the parasitosis of experimental cryptosporidiosis and that this effect was NO mediated. NO has been shown to be cytotoxic to viruses (5), some (4, 25) but not all (8) bacteria, fungi (2), and many eukaryotic cells (12). Activated macrophage-generated NO has been implicated in the stasis and killing of both intracellular (1, 15, 19, 24) and extracellular (23) protozoan parasites. The arginine effect seen in the present experiments both may have been the result of a direct antiparasitic effect of NO, or a NO derivative, as suggested by the in vitro experiments, and may have involved an indirect effect due to arginine enhancement of extrathymic T-cell function. In the nude mouse, dietary arginine has been shown to increase splenic T cells, mononuclear cell blastogenic responses to phytohemagglutinin and concanavalin A, and delayed-type hypersensitivity (11).

The evidence of a NO-mediated anticryptosporidium effect in the present study is not surprising as the administration of anti-gamma interferon antibodies was found to increase oocyst shedding in infected BALB/c mice (22). The antiparasitic effects of gamma interferon- or tumor necrosis factor alpha-activated macrophages have been directly correlated with the release of NO in some intracellular parasites (1, 15). In the nude mouse model, there appeared to be some NO-mediated amelioration of the infection throughout its course that was unmasked by the administration of the NO synthase inhibitor. *Cryptosporidium* infections in steroid-immunosuppressed rodent models develop more rapidly than they do in immunodeficient rodent models (13, 14). Steroid hormones inhibit the induction of NO synthase in macrophages (7), and such inhibition may explain the more rapid onset of cryptosporidiosis in the immunosuppressed rodent.

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REFERENCES

1. **Adams, L. B., J. B. Hibbs, Jr., R. R. Taintor, and J. R. Krahenbuhl.** 1990. Microbiostatic effect of murine-activated macrophages for *Toxoplasma gondii*. Role for synthesis of inorganic nitrogen oxides from L-arginine. J. Immunol. **144:**2725–2729.
2. **Alspaugh, J. A., and D. L. Granger.** 1991. Inhibition of *Cryptococcus neoformans* replication by nitrogen oxides supports the role of these molecules as effectors of macrophage-mediated cytostasis. Infect. Immun. **59:**2291–2296.

2a. **Arrowood, M. J.** Personal communication.

3. **Arrowood, M. J., J. M. Jaynes, and M. C. Healey.** 1991. In vitro activities of lytic peptides against sporozoites of *Cryptosporidium parvum*. Antimicrob. Agents Chemother. **35:**224–227.
4. **Boockvar, K. S., D. L. Granger, R. M. Poston, M. Maybodi, M. K. Washington, J. B. Hibbs, Jr., and R. L. Kurlander.** 1994. Nitric oxide produced during murine listeriosis is protective. Infect. Immun. **62:**1089–1100.
5. **Croen, K. D.** 1993. Evidence for an antiviral effect of nitric oxide. J. Clin. Invest. **91:**2446–2452.
6. **Current, W. L., and L. S. Garcia.** 1991. Cryptosporidiosis. Clin. Lab. Med. **11:**873–897.
7. **Di Rosa, M., M. Radomski, R. Carnuccio, and S. Moncada.** 1990. Glucocorticoids inhibit the induction of nitric oxide synthase in macrophages. Biochem. Biophys. Res. Commun. **172:**1246–1252.
8. **Doi, T., M. Ando, T. Akaike, M. Suga, K. Sato, and H. Maeda.** 1993. Resistance to nitric oxide in *Mycobacterium avium* complex and its implication in pathogenesis. Infect. Immun. **61:**1980–1989.
9. **Gianotti, L., J. W. Alexander, T. Pyles, and R. Fukushima.** 1993. Arginine-supplemented diets improve survival in gut-derived sepsis and peritonitis by modulating bacterial clearance. Ann. Surg. **217:**644–654.
10. **Heine, J., H. W. Moon, and D. B. Woodmansee.** 1984. Persistent *Cryptosporidium* infection in congenitally athymic (nude) mice. Infect. Immun. **43:**856–859.
11. **Kirk, S. J., M. C. Regan, H. L. Wasserkrug, M. Sodeyama, and A. Barbul.** 1992. Arginine enhances T-cell responses in athymic nude mice. J. Parenter. Enteral Nutr. **16:**429–432.
12. **Kroncke, K.-D., H.-H. Brenner, M.-L. Rodriguez, K. Etzkorn, E. A. Noack, H. Kolb, and V. Kolb-Bachofen.** 1993. Pancreatic islet cells are highly susceptible towards the cytotoxic effects of chemically generated nitric oxide. Biochim. Biophys. Acta **1182:**221–229.
13. **Leitch, G. J., and Q. He.** 1994. Putative anticryptosporidial agents tested with an immunodeficient mouse model. Antimicrob. Agents Chemother. **38:**865–867.
14. **Lemeteil, D., F. Roussel, L. Favennec, J.-J. Ballet, and P. Brasseur.** 1993. Assessment of candidate anticryptosporidial agents in an immunosuppressed rat model. J. Infect. Dis. **167:**766–768.
15. **Liew, F. Y., Y. Li, and S. Millott.** 1990. Tumor necrosis factor (TNF-α) in leishmaniasis. II. TNF-α-induced macrophage leishmanicidal activity is mediated by nitric oxide from L-arginine. Immunology **71:**556–559.
16. **McGowan, I., A. S. Hawkins, and I. V. D. Weller.** 1993. The natural history of cryptosporidial diarrhea in HIV-infected patients. AIDS **7:**349–354.
17. **Mead, J. R., M. J. Arrowood, R. W. Sidwell, and M. C. Healey.** 1991. Chronic *Cryptosporidium parvum* infection in congenitally immunodeficient SCID and nude mice. J. Infect. Dis. **163:**1297–1304.
18. **Moncada, S., R. M. J. Palmer, and E. A. Higgs.** 1989. Biosynthesis of nitric oxide from L-arginine; a pathway for the regulation of cell function and communication. Biochem. Pharmacol. **38:**1709–1715.
19. **Rockett, K. A., M. M. Awburn, W. B. Cowden, and I. A. Clark.** 1991. Killing of *Plasmodium falciparum* in vitro by nitric oxide derivatives. Infect. Immun. **59:**3280–3282.
20. **Shaffer, J. E., B.-J. Han, W. H. Chern, and F. W. Lee.** 1992. Lack of tolerance to a 24-hour infusion of S-nitroso N-acetylpenicillamine (SNAP) in conscious rabbits. J. Pharmacol. Exp. Ther. **260:**286–293.
21. **Unger, B. L. P., J. A. Burris, C. A. Quinn, and F. D. Finkelman.** 1990. New mouse models for chronic *Cryptosporidium* infection in

immunodeficient hosts. Infect. Immun. **58:**961–969.

22. **Unger, B. L. P., T.-C. Kao, J. A. Burris, and F. D. Finkelman.** 1991. *Cryptosporidium* infection in an adult mouse model. Independent roles for IFN-γ and $CD4^+$ T lymphocytes in protective immunity. J. Immunol. **147:**1014–1022.
23. **Vincendeau, P., and S. Daulouede.** 1991. Macrophage cytostatic effect on *Trypanosoma musculi* involves an L-arginine dependent mechanism. J. Immunol. **146:**4338–4343.
24. **Vincendeau, P., S. Daulouede, B. Veyret, M. L. Darde, B. Bouteille, and J. L. Lemesre.** 1992. Nitric oxide-mediated cytostatic activity on *Trypanosoma brucei gambiense* and *Trypanosoma brucei brucei*. Exp. Parasitol. **75:**353–360.
25. **Zhu, L., C. Gunn, and J. S. Beckman.** 1992. Bactericidal activity of peroxynitrite. Arch. Biochem. Biophys. **298:**452–457.