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# CRYPTOSPORIDIOSIS IN THE YOUNG AND MALNOURISHED HOST
# FINAL REPORT

This project resulted in the completion of three separate subprojects. These subprojects are
1. The role of host factors in the infection of neonatal mice with Cryptosporidium parvum.
2. Vitamin A deficiency exacerbates Cryptosporidum parvum infection in immunodeficient and immunosuppressed mice.
3. The lectin Jacalin inhibits the establishment of a Cryptosporidum parvum infection in immunodeficient and neonatal mice.

**The role of host factors in the infection of neonatal mice with Cryptosporidium parvum.**

In animals other than primates the intestinal parasitic disease, cryptosporidiosis, can only be produced in neonates or in immunodeficient or immunosuppressed animals. In animals such as rodents and calves there is only a short period shortly after birth during which the animals can be infected. Thereafter the parasite cannot establish an infection. Similarly there is a period beyond which an active infection is no longer supported and the parasite is cleared from the intestine. Understanding the mechanisms by which this age dependent resistance to infection or expulsion of parasite occurs would provide clues as to the mechanisms that might be exploited to treat this parasitic disease in immunodeficient and undernourished humans who are at risk.

The course of C. parvum infection is neonatal BALB/C mice has been extensively studied. The magnitude and location of the infection has been followed histologically. Basically when animals were orally infected at 4 days of age with 40,000 C. parvum the infection peaked in the ileum and colon by day 14 and was completely clearer by day 30, with clearing occurring first in the colon. There was the suggestion of a light infection in the jejunum of some 8 and 10 day old animals. Fecal oocyst shedding was assessed by a commercial indirect immunofluorescent assay and an oocyst shedding score developed. This scoring method has been found to correlate well with the degree of infection in the ileum but not the colon.

As the ileum appeared to be the major site of infection in these neonatal animals, we concentrated most of our efforts in studying this area of the gastrointestinal tract. The ileal villus:crypt ratio was found to decrease significantly as the infection progressed and to return to control animal values as the infection was resolved. At the time when the infection seemed to peak (day 14), there was evidence of villus epithelial cell damage that exceeded what would be expected from the number of detected parasites. This was surprising given that the crypts, not the villi, were the major site of infection.

As others have shown, we found that in the neonatal mouse there is a window of about 25 days when the animals can be infected using an oral administration of C. parvum oocysts. Thereafter there was evidence of a very light infection lasting only 2 - 3 days. This light infection was detectable in some animals as oocyst shedding, but no animal exhibited a histologically identifiable infection.

We had previously found that experimental cryptosporidiosis was ameliorated by feeding adult immunodeficient mice an arginine-rich diet. This observation suggested that nitric oxide plays some role in determining the course of this parasitic infection. In the present

study we have used an immunohistochemical method to assess the intestinal inducible nitric oxide synthase (iNOS) in uninfected and infected animals of different ages. The basal iNOS levels within the mucosal epithelium of uninfected animals were much higher in the duodenum and jejunum than in the colon, and the levels within the ileum were often undetectable. In all segments of the small intestine the iNOS was greatest in the more differentiated enterocytes on the apices of the villi. In infected animals there appeared to be a reduction in the enterocyte iNOS levels in the ileum. This was consistent with the fact that the parasite infection reduced the differentiated enterocytes on the villi, thereby reducing villus height and epithelial iNOS levels. The fact that the two intestinal segments with the highest basal epithelial iNOS levels were also the areas that would not support parasite infection suggested that NO played a role in determining which regions of the intestine could be infected.

In order to determine if host intestinal mucosa-derived NO was an important factor in determining the susceptibility of neonatal mouse intestine to experimental cryptosporidiosis, we have studied the effects of the NOS inhibitor L-NAME on such infections. A number of different protocols were used in which L-NAME (1, 10 or 100 mg/Kg/day) was administered orally to infected mice to determine if this agent had any effect on the severity or clearing of an existing infection. In general the two higher doses of this agent caused a significant increase in fecal oocysts shedding score and in ileal and colonic parasite load (assessed by a subjective infection score). In no case did this agent cause the appearance of infection in the duodenum and jejunum. At the time when the infection was being cleared in control animals (days 24 and 30), significantly more animals exhibited infection in the two higher L-NAME dose groups. However, when uninfected 30 day old animals were treated with the highest L-NAME dose for 4 days prior to and following the oral administration of 40,000 C. parvum oocysts, 4 of 6 animals exhibited a histologically identifiable modest ileal infection while none of the a group of 6 vehicle control animals displayed histological or fecal oocyst evidence of infection.

Infected control and 100 mg/Kg/day L-NAME-treated animals were given an intraperitoneal injection of 100 $\mu$g anti-gamma interferon antibody on day 10 or on days 10 and 18 when experiments that were carried beyond day 18. Anti-gamma interferon antibody alone significantly increased the fecal oocyst shedding and ileal and colonic infection scores and increased the number of animals still infected on day 24. Animals given both the high dose of the NOS inhibitor and the anti-gamma interferon antibody showed a massive increase in fecal oocyst shedding, a significant increase in colonic infection score and in the ileum a significant shortening of the villi and almost complete denuding of the epithelium.

Taken together, these data indicate that gamma interferon and host NO make a contribution to holding down the intestinal parasitosis in the ileum and colon, and play a role in the eventual clearing of the parasite. They do not, however, indicate that these factors play a role in determining if an area of the intestine will be susceptible to infection by this protozoan parasite.

**Vitamin A deficiency exacerbates Cryptosporidium parvum infection in immunodeficient and immunosuppressed mice.**

Vitamin A deficiency has long been known to result in increased death rates in children exposed to a variety of childhood diseases. The immunodeficiencies known to accompany

this vitamin deficiency may contribute to the high susceptibility of AIDS patients to opportunistic infections. AIDS patients and malnourished children are particularly at risk from cryptosporidiosis. To date only one study has been performed in which vitamin A deficiency and cryptosporidiosis have been studied together. In that study neonatal cryptosporidiosis was found to reduce vitamin A stores in neonatal pigs. Unfortunately while rodents provide the easiest models for cryptosporidiosis, it is very difficult to produce vitamin A deficiency in these species.

Adult athymic nude mice can be infected with C. parvum. Such infections last for the remaining life of the immundeficient animals (6-18 months). Uninfected animals and animals infected for 3 months were each divided into two groups, one of which was placed on a control diet and the other on a vitamin A deficient diet (Purina Mills) for 50 days. During this period all animals were weighed 3 times a week and any deaths recorded. There was no weight loss or deaths in either of the uninfected animals. However, 4 of 6 of the vitamin A deficient infected animals, and 2 of 6 of the control infected animals died in the 50 day period. Furthermore, in the week preceding the death of the two control animals, they both lost less than 20% of their body weight. On the other hand, all of the vitamin A deficient diet animals that died lost in excess of 25% of their body weight before death.

Another two groups of athymic nude animals were placed on a control and on a vitamin A deficient diet for 50 days prior to and following infection by the oral administration of 50,000 C. parvum oocysts. Fecal oocyst shedding was followed for the next three weeks. The animals on the vitamin A deficient diet shed significantly more oocysts during the study period and at the time of sacrifice the ileal infection scores were also significantly greater in the deficient diet group. In a similar experiment animals on the control diet were divided into two groups. One group was infected and fecal oocyts shedding followed as before. On day 1 postinfection and at weekly intervals thereafter the animals were given 1 mg vitamin A (retinal) administered subcutaneously in corn oil. In the other group the animals were given the weekly injection of the vehicle alone. There was no difference between the two groups of animals in either their daily oocyst shedding or in the ileal infection score at the time of sacrifice.

A group of immunocompetent adult male BALB/C mice were immunosuppressed with 50mg/Kg prednisalone administered subcutaneously for four successive days. On the fourth day the animals were given 50,000 Cryptosporidium oocysts per os and the fecal oocyst excretion followed for 4 days. When these experiments were performed on animals that were either given the vitamin A or vehicle as above on the first and last days of prednisalone treatment the animals given the vitamin A had significantly lower shed oocysts scores than did the vehicle control animals and the ileal infection scores on day 5 postinfection were also significantly lower in this group. Thus vitamin A appeared to partially reverse the prednisalone immunosuppression in these experiments. Vitamin A is known to be both an immunomodulator and an antioxidant. To determine if the effect seen in the immunosuppression experiments was due to the antioxidant properties of vitamin A, two other antioxidant vitamins were used in a similar experiment. In one group of animals vitamin E (1 mg) was administered orally at the onset of the prednisalone administration and at the time the oocysts were administered. In another group, animals were given 10 mg ascorbic acid orally in two divided doses daily after the oocysts were administered. In these experiments shed oocyst excretion scores were followed until the parasite had been cleared.

Neither the vitamin E nor the ascorbic acid animals differed from controls in either the daily shed oocysts excretion score, or in the time at which they appeared to clear the parasite. Thus, these data support the hypothesis that vitamin A, functioning as an immunomodulator, is capable of ameliorating the magnitude of infection with C. parvum under some conditions. Similarly, inducing a vitamin A deficiency in immunodeficient animals results in an exacerbation of the parasite-associated disease.

**The lectin jacalin inhibits the establishment of a Cryptosporidium parvum infection in immunodeficient and neonatal mice.**

Several protozoan parasites have been shown to attach to target cells by a surface lectin that identifies some host cell sugar. Perhaps the best characterized of these interactions is that of Entamoeba histolytica that employs a N-acetylgalactosamine (galNAC)/galactose (gal) recognizing lectin. Recently a surface lectin has been implicated in the attachment of C. parvum sporozoites to target cells. The attachment of sporozoites and oocysts to erythrocytes can be inhibited by a number of gal or galNAC-containing sugars. We have surveyed a number of glucose, galactose, N-axcetylglucosamine and N-acetylgalactosamine binding lectins for their ability to affect the progress of experimental cryptosporidiosis in several mouse models. Of the panel of lectins studied, the Brazilian jackfruit lectin, jacalin, was found to be the most effective inhibitor of experimental cryptosporidiosis. This lectin bind to both gal and galNAC.

To demonstrate a lectin effect on the course of experimental cryptosporidiosis neonatal BALB/C mice were infected on day 4 with 50,000 C. parvum oocysts and the resulting infection assessed 4 days later by determining the colonic content oocysts score and the ileal and colonic infection scores. It was assumed that the major site of any lectin effect would be to inhibit the attachment of excysting sporozoites to target enterocytes. In order to occupy parasite attachment sites, 200 $\mu$g of the test lectin was administered orally in 0.2 ml saline to the animals 4 hour prior to the oocysts. When this was done using jacalin, a significant inhibition was observed in the colonic oocyst load. The ileal infection score method did not detect any jacalin effect, and both control and lectin-treated animals exhibited a maximal infection score. When the jacalin was administered 4 hours prior to and 24 hours after the oocysts administration both the colonic oocysts load and the ileal infection score were significantly reduced. When the jacalin was administered 24, 48 and 72 hours after the oocysts administration there was a reduction in the ileal infection score that was not statistically significant. These results were interpreted to indicate that lectin administered prior to the oocysts blocked the infection caused by some sporozoites. The fact that we were unable to demonstrate a significant inhibition in infection when the lectin was administered 24, 48 and 72 hours after oocysts administration suggested either that the lectin was ineffective at blocking the entry of the merozoite stage of the parasite or that the infection was so extensive as to not be affected by the lectin. The same experiment was performed with the adult nude mouse model administering 400 $\mu$g jacalin 4 hours prior to or 24, 48 and 72 hours after the oocysts. The same results were obtained as with the neonatal animals.

Two types of experiments were performed to determine if jacalin could be used to identify C. parvum attachment sites in the mouse intestine. In the first experiment excised loops of neonatal mouse intestine were filled with phosphate buffered saline solutions containing 100 $\mu$g/ml FITC-labelled jacalin. The loops were incubated at room temperature

for 30 minutes, washed extensively with saline to remove any non-adsorbed lectin and then incubated in saline solutions containing varying concentrations of galactose or N-acetyl galactosamine. The desorbed fluorescent jacalin was then measured fluorometrically and normalized to intestinal loop weight. Paraffin-embedded sections of neonatal and adult mouse intestines were deparaffinized, stained with FITC-labelled jacalin, mounted and examined by confocal microscopy. Both types of experiments indicated that there was an abundance of jacalin binding sites in both the mucus and at the brush border in duodenum, jejunum, ileum and proximal colon of both neonatal BALB/C and adult athymic nude mice. If jacalin had bound only target cell C. parvum attachment sites, then one might have expected that those areas such as the duodenum and jejunum of neonatal BALB/C and adult nude mice would not have adsorbed the lectin. The data therefore suggest that jacalin had its effect by binding to a large number of target cell sites, including those to which the sporozoites and perhaps merozoites attached.

In another experiment bile acid stimulated-oocyst excystation was studies in the presence and absence of jacalin. Similarly, excysted sporozoites were exposed to jacalin and their viability compared with that of unexposed parasites. In both cases we were unable to demonstrate a jacalin effect over the concentration range of 1 - 100 $\mu$g/ml. However, when the jacalin was included in the excysting assay and the viability of the freshly excysted sporozoites was measured, we found that jacalin concentrations as low as 10 $\mu$g/ml caused significant parasite killing. Freshly excysted sporozoites are known to be covered with a glycoprotein coat that is used in their gliding motility and is shed as motility progresses. Presumeably this coat is the site of jacalin binding in the excysting sporozoites.

Thus, while we have demonstrated that this lectin is cap[able of reducing the severity of experimental cryptosporidiosis, we have not demonstrated that this effect is only due to lectin competing with invading parasite stages for the target cell attachment sites. Using lectins to study the course of this parasite infection has two potential rewards. First this type of study aids in understanding the role of parasite and host cell surface glycoproteins in the infection process, and second, it raises the possibility that dietary lectins may have potential use as a "low-tech" method of ameliorating the signs and symptoms of cryptosporidiosis in the field.

Some of these data have been reported on at national meetings and three manuscripts are in preparation.

**Presentations:**

Leitch, G.J. and He, Q. Role of host nitric oxide in experimental cryptosporidiosis in a nude mouse model. The 47th Annual Meeting of the Society of Protozoologists 3rd Workshop on Opportunistic Protozoan Pathogens, Cleveland, OH, June 24-29, 1994.

Leitch, G.J., He, Q., Russell, S.A. and Rawls, R.A. Effect of vitamin A and vitamin A deficiency on experimental cryptosporidiosis. The 44th Annual Meeting of the American Society of Tropical Medicine and Hygiene. San Antonio, TX, November 17-21, 1995.

Leitch, G.J., He, Q., Soyoola, E.O. and Johnson, T.L. Jacalin reduces experimental cryptosporidiosis in the mouse. The 96th General Meeting of the American Society for Microbiology, New Orleans, LA, May 19-23, 1996.

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