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Giardia CWP2: determining its immunogenicty and its potential as a candidate for vaccine purposes against giardiasis

By

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A Thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

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March 2000

ABSTRACT

In this study, we determined the immunogenicity of *CWP2* and its potential as a vaccine candidate against giardiasis. *CWP2* was expressed as a recombinant protein with an hexa-histidine affinity tag and was isolated from inclusion bodies. When BALB/c mice were immunized with *CWP2*, a specific IgA was detected in the feces. When mice were immunized with *CWP2* + cholera toxin, as an adjuvant, IgA in the feces, and IgA, IgG1, and IgG2a in the serum, all specific to *CWP2*, were detected. Also, CD-1 mice were infected with *G. muris* and presence of specific IgA antibodies to *CWP2* were detected in the feces. This result indicated that *CWP2* was recognized by the immune system in a natural infection. IL-4 and IL-5 were released from Peyer's patches (PP) and mesenteric lymph nodes (MLN) cells when stimulated with concanavalin A. In spleen cells, IFN-γ, IL-4, and IL-5 were released when stimulated with concanavalin A. However, in PP, MLN and spleen cells, the levels of cytokines were barely detectable when stimulated with *CWP2*. The presence of IgG2a (Th1), IgA and IgG1 (Th2) as the production of IFN-γ (Th1), IL-4 and IL-5 (Th2) confirmed that *CWP2*, when presented orally to mice, stimulates both a Th1 and Th2 type immune response, locally and systemically.

ABREGE

Dans cette étude, l'immunogénicité de *CWP2* et son potentiel en tant que protéine candidate pour le développement d'un vaccin contre la giardiose ont été évalués. La protéine *CWP2* a été exprimée sous forme de protéine recombinante avec un signal hexa-histine et a été isolée des corps d'inclusions. Quand des souris BALB/c ont été immunisées avec *CWP2*, des IgA spécifiques ont été détectés dans les fèces. Cependant lorsque les souris ont été immunisées avec *CWP2* + "cholera toxin", on a détecté des IgA dans les fèces et des IgA, IgG1 et IgG2a dans le sérum, tous spécifiques à *CWP2*. Des souris CD-1 ont été infectées avec *G. muris* et la présence d'anticorps IgA spécifique à *CWP2* a été détectée dans les fèces. Ce résultat indique que *CWP2* est reconnu par le système immunitaire lors d'une infection naturelle. Lorsque les cellules des plaques de Peyer (PP) et des ganglions lymphatiques mésentériques (GLM) ont été stimulées par la concanavaline A, des IL-4 et IL-5 ont été relâchés tandis que lorsque les cellules de la rate ont été stimulées par la concanavaline A, IFN-γ, IL-4 and IL-5 ont été relâchés. Cependant, lorsque les cellules des PP, des GLM et de la rate ont été stimulées par *CWP2*, les niveaux de cytokines étaient à peine détectables. La présence de IgA et IgG1 (Th2) et de IgG2a (Th1) ainsi que de IFN-γ (Th1), IL-4 et IL-5 (Th2) confirme que lorsque l'antigène *CWP2* est présenté oralement aux souris, il stimule une réponse immunitaire locale et systémique de type Th1 et Th2.

ACKNOWLEDGEMENTS

I would like to thank my thesis supervisor Dr. G. M. Faubert for his guidance and support throughout my Master's project. Dr. Faubert gave me the opportunity to present my results at the American society of Parasitologists 47th Annual meeting in Monterey, California in July 1999; this was greatly appreciated. I wish to thank the other members of my advisory committee: Dr. K. Chadee and Dr. W. Lapp, for their advice and suggestions.

I would also like to thank my laboratory partner, Peter Lee, for his assistance and friendship. Thanks also to Shawn Mohammed, a technician who worked in the lab, for his help during my research project. Specials thanks to Dr. K. Nagakaki, a visiting researcher, for his assistance and stimulating discussions in the laboratory. I am also indebted to many students and staff for their valuable help, support and friendship: G. Bingham for maintaining the animals used in my research, Dr. M. Scott for being a great teacher, Sylvie Labreque for all her help in the laboratory and Shirley Mongeau for her secretarial services.

I would like to express my gratitude to the Institute of Parasitology of McGill University and Dr. Faubert for providing me with financial help.

Lastly, I want to thank my parents and my sister, Marie-Eve, for their love, friendship and encouragement.

The data of the experimental work reported in this thesis was done by R. Larocque.

in partial fulfillment of the requirements for the degree of Masters of Science.

Dr. G. M. Faubert acted as my supervisor.

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REVIEW OF LITERATURE

LIST OF ABBREVIATIONS

Ab	Antibody
ABTS	2.2-azubi-di (3-ethylbenzthiazoline-6-sulphonic acid)
ADCC	Antibody-dependant cell-mediated cytotoxicity
Ag	Antigen
APC	Antigen presenting cell
BiP	Binding Protein
BSA	Bovine Serum Albumin
C	Celsius
ca	Circa
Con A	Concanavalin A
CT	Cholera Toxin
CRP	Cystein-rich Protein
CWP	Cyst Wall Protein
ECL	Enhanced Chemi-Luminescent
ELISA	Enzyme-linked Immunosorbent Assay
ESV	Encystation Specific Vesicles
FBS	Fetal Bovine Serum
Fig.	Figure
g	Gravity
h	Hour
GALT	Gut-associated Lymphoid Tissues
IEL	Intraepithelial Lymphocytes
IFN-γ	Interferon-gamma

Ig	Immunoglobulin
IL	Interleukin
IPTG	Isopropyl-β-D-Thiogalacto-Pyranoside
KD	Kilodalton
LB	Luria-Bertani
LPL	Lamina Propria Lymphocytes
LRR	Leucine-rich Repeat
M cells	Microfold Cells
mAb	Mononclonal Antibody
MLN	Mesenteric Lymph Node
NK cells	Natural Killer Cells
O.D.	Optical Density
OPD	o-Phenylenediamime
PBS	Phosphate Buffered Saline
PMSF	Phenylmethyl Sulfonylfluoride
PP	Peyer's Patches
r-*CWP2*	Recombinant *CWP2*
ROI	Reactive Oxygen Intermediate
RT	Room Temperature
SC	Secretory Component
SEM	Standard Error of the Mean
sem	Scanning Electron Microscopy
S-Ig	Secretory Ig
Tc	T cytotoxic

Th	T helper
TP-S-1	Trypticase Panmede Serum #1
TT	Tetanus Toxoid
TYI-S-33	Trypticase Yeast Extract Iron Serum #33
VSP	Variant Surface Protein

INTRODUCTION

Giardiasis is the most frequently reported human parasitic infection in Canada. Manifestation of the disease varies from asymptomatic carriage to severe diarrhea and malabsorption. The disease is caused by a protozoan parasite called *Giardia lamblia*. The parasite exists in two forms: cyst and trophozoite, but it is transmitted by cyst. Transmission can occur by personal contact with infected people or drinking contaminated water. There is evidence that giardiasis is a zoonosis (Moorehead *et al.*, 1990; Buret *et al.*, 1990). Farm animals play a major role in the contamination of surface water (LeChevalier *et al.*, 1991; Buret *et al.*, 1990; Ruest *et al.*, 1998). The passage of Giardia from one host to a new host requires encystation of trophozoites. Therefore, encystation becomes an interesting target for controlling the spreading of the infection.

Campbell and Faubert (1994a) developed a monoclonal antibody (mAb) 8C5.C11, which when added to encysting culture of *Giardia*, reduced the formation of cysts in vitro by 60%. Similar reduction percentages were also observed *in vivo* when a crude extract of encysting cells was used to actively immunize mice. When the immunized mice were challenged with live cysts, they released fewer cysts in their feces when compared to non-immunized mice (unpublished data). These results show that the antigen binding to the mAb developed by Campbell and Faubert (1994a) is a possible candidate for the development of a vaccine directed against the infective stage of the parasite. One of those antigens was found to be cyst wall protein 2 (*CWP2*) which was shown to react with mAb 8C5 (unpublished data). *CWP2* is a 39 KD cysteine-rich protein required for the formation of the cyst wall. The gene coding for *CWP2* was cloned using a mAb to cyst wall antigen (Lujan *et al.*, 1995).

Because the immunogenicity of *CWP2* is unknown, studied the immune response to this cyst antigen in mice. For this purpose, BALB/c mice were immunized orally with *CWP2* using cholera toxin as an adjuvant.

OBJECTIVES

The objectives of this Masters research project were to:

1) Express *CWP2* as a recombinant His-tag protein in an *E. coli* bacterial over-expression system.

2) Determine the solubility of *CWP2* with a solubility assay.

3) Isolate *CWP2* from inclusion bodies.

4) Study the immune response to *CWP2* in immunized BALB/c mice.

5) Determine the presence of *CWP2* antibodies in the feces of CD-1 mice infected with *G. muris*.

REVIEW OF LITERATURE

1.Classification

Trophozoites of the protozoan *G. lamblia* were first described in 1681 when Antony van Leeuwenhoek developed his microscopes (Meyer. 1990). He observed the protozoan from his own diarrhetic stool. However. the credit of the discovery of the flagellate *Giardia* was given to the Czech physician. Vilem Lambl in 1859. Twenty years later. the cyst form of the organism was described by Grassi (1879). Early workers believed that the protozoa classified in the genus *Giardia* were host specific (Hegner. 1926). Therefore. species were initially assigned by the host from which they were isolated. which gave rise to more than 40 *Giardia* species. However. Filice (1952) considered the concept of strict specificity for *Giardia* unreliable and proposed a new classification. Filice used the morphology of the trophozoite median body to classify the *Giardia* species into three different groups. The trophozoites which infects tadpoles and frogs have a long tear-drop shape median body and were given the name *G. agilis* (Fig. 1a). The trophozoites found in rodents and birds have 2 small round median bodies and were given the name *G. muris* (Fig. 1b). The classification of *G. lamblia* is presented in table 1. The trophozoites found in humans and other mammals have a claw hammer shaped median body and were given the name *G. duodenalis* (Fig. 1c).

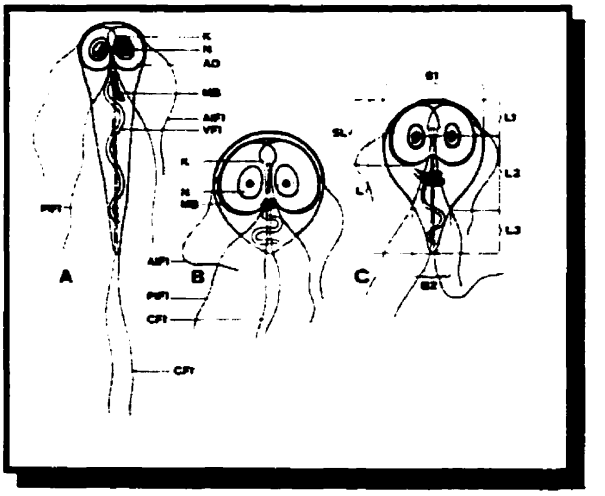

Figure 1. The three morphological types of *Giardia*. View of trophozoites of *G. agilis* from amphibians (A), *G. muris* from mice (B), and *G. duodenalis* from man (C). (From Kulda and Nohynkova. 1978)

In addition to the three aforementioned morphological groups, two other species of *Giardia* isolated from birds have been described recently. *G. psittaci* was isolated from budgeriran (Erlandsen and Bemrick, 1987) and *G. ardea* from the great blue heron (Erlandsen *et al.*, 1990). Even if both species share many of the characteristics of *G. duodenalis*, they were found to be different from the trophozoites of the *duodenalis* group when examined by electron microscopy. It is likely that new *Giardia* species will be described in the future. In this thesis, *G. lamblia* will be used to describe the human type of *Giardia*.

Table 1. Classification of *G. lamblia*

Phylum	*Protozoa*	(Honingberg, 1964)
Subphylum	*Sarcomastigophora*	(Honingberg and Balamuth, 1963)
Superclass	*Mastigophora*	(Diesing, 1866)
Class	*Zoomasigophora*	(Calkins, 1909)
Order	*Diplomonadida*	(Wenyon, 1926)
Family	*Hexamitidae*	(Kent, 1980)
Subfamily	*Octomitinae*	(Von Prowazek, 1904)

(Reviewed in Meyer, 1990; Roberts and Janovy, 1996)

2. Biology of *Giardia lamblia*

a) Morphology

i) Trophozoite

G. lamblia exists in two forms: the trophozoite which is the vegetative stage and the cyst which represents the infective stage.

The trophozoite (Fig. 1a) measures approximately 12 to 15 µm in length and 5 to 9 µm in width. It is a bilaterally symmetrical structure resembling a teardrop. It is characterized by a

convex dorsal surface and a concave ventral surface. The ventral surface of the trophozoite is mostly occupied by the ventral disc which is an attachment organelle that enables it to adhere to the host cell. It is a rigid structure reinforced by microtubules and fibrous ribbons, surrounded by a flexible and contractile striated rim of cytoplasm. The adhesive disc of *Giardia* easily distinguishes this organism from other flagellates. The trophozoite has two nuclei which are found in the anterior half of the organism. A pair of flagella are located at each of the following positions: posterior, anterior, ventral and caudal. A pair of claw hammer shaped median bodies are found in the posterior portion of the organism. The median bodies, which are unique to the genus *Giardia*, are composed of microtubules with unknown function. The trophozoite cannot survive outside of its host.

ii) Cyst

The cyst (Fig. 2) is ovoid in shape, measures approximately 7 to 10 µm and is surrounded by a cyst wall of 0.3 µm (Sheffield and Bjorvatn, 1977). The outer portion of the wall consists of a filamentous layer of 7 to 20 filaments (Erlandsen *et al.*, 1989), whereas, the inner membranous portion consists of two membranes separated by a thin layer of cytoplasm (Erlandsen *et al.*, 1990). Jarrol *et al.* (1989) believe that galactosamine is the major sugar of the outer cyst wall. On the other hand, it has been reported that the cyst wall is made of chitin (Ward *et al.*, 1985). Most cysts contain two live trophozoites. The cyst is water resistant and can survive in the environment for more than 20 days (Faubert *et al.*, 1986). The resistance of the cyst to harsh environmental conditions is due to its rigid filamentous structure which also ensures the viability of the trophozoites located inside.

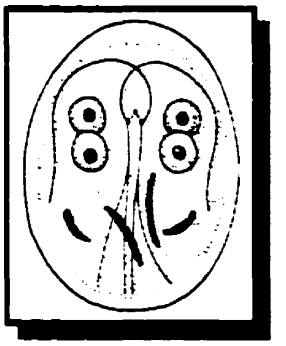

Figure. 2. The cyst structure (From Roberts and Janovy, 1996)

b) Life cycle

The life cycle of *G. lamblia* is simple and direct. Infection is initiated by ingestion of cysts. Exposure of cysts to gastric acid during their passage through the stomach triggers excystation.

After emerging from the cysts, the trophozoites colonize the duodenum and the jejunum. They rapidly multiply by binary fission and attach to the gut epithelium with their adhesive disc. As trophozoites move along the small intestine and enter the colon, encystation may occur, but not all trophozoites are induced to encyst (Campbell and Faubert, 1994). During the encystation process, trophozoites lose their flagella while the cytoplasm condenses and secretes a hyaline cyst wall. Cysts are then passed in feces and represent the infective stage for the next host.

3. Epidemiology of *Giardia lamblia*

G. lamblia is the most commonly isolated intestinal parasite in Canada and the United States and is especially prevalent in children in developing countries. It has a worldwide distribution with a prevalence of about 7.2% in humans (Belding, 1965). When using microscopy, the rates of detection for the parasite after examination of one specimen vary between 2 and 5 % in industrialized countries and between 20 and 30% in developing countries (Marshall *et al.*, 1997). The cysts are highly infectious for humans; ingestion of only 10 viable cysts can result in an infection (Rendtorff, 1954). The cyst can also survive in clean water for more than 20 days (Faubert *et al.*, 1986). Because of the ability of *Giardia* cysts to survive in freshwater, *G. lamblia* is known to be the most common cause of epidemic waterborne diarrheal disease (Adam, 1991). Epidemics have usually been associated with inadequate treatment of contaminated water supplies. Wallis *et al.* (1996) have recently studied the prevalence of *Giardia* cysts isolated in running surface water in Canada. Cysts were found in 73% of raw sewage samples, in 21% of surface water samples and in 18.2% of chlorinated filtered water samples. There is compelling evidence that many wild and domestic animals carry *Giardia* species and thus could be important reservoirs. Beavers were implicated as the source of a giardiasis outbreak in British Colombia in 1986 (Moorehead *et al.*, 1990), and it was assumed that muskrats, which were found to be *Giardia*-positive in Germany,

could contaminate water bodies (Karanis *et al.*, 1996). Domestic cats and dogs have also been shown to carry *Giardia* (Leib *et al.*, 1999) and could be a potential source of infection for their owners. Evidence suggested that giardiasis is a zoonosis in domestic ruminants, especially in sheep and cattle (Buret *et al.*, 1990). It has been reported that worldwide, infection rates of *Giardia* in cattle, range from 1% to 51.6% (Xiao, 1994). A Canadian study that looked at 6 different farms in southern Alberta, found that the overall infection rate for *Giardia* was 10.4% (Buret *et al.*, 1990). In a more recent study, Ruest *et al.* (1998) looked at the prevalence of *Giardia* on dairy farms in Quebec. They found that 45.7% of the cattle farm samples were found to be positive for *Giardia*. A survey of 66 surface water treatment plants in 14 states and in one Canadian province showed that surface water receiving agricultural effluent had a higher density of *Giardia* cysts than protected water sheds (LeChevalier *et al.*, 1991). This suggests that cattle may play a major role as a reservoir of the infection and thus, play a role in the contamination of surface water that is used for drinking and recreational purposes. Direct person to person contact is another major mechanisms by which the disease is transmitted. This occurs especially in day care centers where the crowding, the sharing of toys and food contribute to a high (35%) prevalence of giardiasis in children (Woo and Patterson, 1986). An increased prevalence of giardiasis in homosexual men has also been observed and is probably the result of fecal-oral transmission (Schmerin *et al.*, 1978). It has been suggested that some cysts are able to survive in food and contribute to foodborne transmission of giardiasis, however, this mode of transmission is a less common source of giardiasis (Petersen *et al.*, 1988).

4. *In vitro* Cultivation

Culture media capable of supporting the non-axenic growth of most parasite flagellates of the alimentary canal for up to five weeks, including *G. lamblia*, were available as early as 1921(Meyer, 1990). However, it was not until 1960, that Karapetyan reported that *G.lamblia* had

been cultured symbiotically with chick fibroblasts and *Candida guilliermondii*, for seven months. He was subsequently able to sustain *G. lamblia*, isolated from a rabbit, for up to five months in a medium where *Saccharomyces* yeast replaced *Candida* (1962). However, Karapetyan (1962) reported numerous unsuccessful attempts to axenize the culture of *G.lamblia*.

Meyer (1970) reported the first axenic cultivation of *Giardia* trophozoites isolated from rabbits, chinchillas and cats. The *Giardia* cultures were axenized by separating them from yeast (*S. cerevisiae*) in a U-shaped culture tube. In 1976, Meyer described axenic cultivation of trophozoites from humans. Visvesvara (1980), axenically cultivated trophozoites by adapting the Diamond's TP-S-1 media, which was originally formulated for the cultivation of *Entamoeba histolytica*. TP-S-1 medium contains trypticase, panmede and bovine serum. Due to difficulties in obtaining panmede, Diamond *et al.* (1978) developed a new medium for the axenic cultivation of *E. histolytica*: TYI-S-33 (trypticase, yeast extract, iron, serum). Subsequently, Gillin and Diamond (1981) succeeded in growing *Giardia* trophozoites in filter-sterilized TYI-S-33 medium. The reducing agent, L-cysteine, appears to be an essential reagent for a successful growth of trophozoites *in vitro*. The low tension of oxygen created by L-cysteine mimics the small intestine habitat, implying that *Giardia* trophozoites grow under anaerobic conditions *in vivo*. It is worthwhile to mention that only the trophozoites of *G.lamblia* species have been successfully cultured *in vitro*, and even some isolates of *G. lamblia* cannot be grown in TYI-S-33 (Meloni and Thompson, 1987; Thompson *et al.*, 1990).

5. *In Vitro* Encystation of *Giardia*

a) Stimulus for encystation

In vitro encystation of *G. lamblia* trophozoites was reported for the first time by Gillin *et al.*(1987). They attempted to mimic the intestinal conditions *in vitro* that allow for the encystment of the trophozoites *in vivo*. In mice, *Giardia* trophozoite encystation occurred in the mid to lower

jejunum. In their natural niche, trophozoites live in the upper small intestine attached to the epithelial cells of the gut underneath a mucus layer which sequesters them from the intestinal fluid. However, when the trophozoites are swept to the lower duodenum and jejunum by the peristaltic movement of the intestine, they are exposed to high concentrations of bile salts and products of lypolysis. These conditions were mimicked *in vitro*. Therefore, Gillin *et al.*(1987) added bile salts glycodeoxycholate and oleic acid to the TYI-S-33 growth medium. After six days of incubation at 37^{0}C, they found that 9.1% of trophozoites had encysted. In a subsequent study, Gillin *et al.* (1988) showed that optimal encystation was observed when primary bile salts, glycocholate with myristic acid were added to the medium. Moreover, the pH of the medium had to be adjusted to 7.8 to mimic the pH of the jejunum. In other studies, it was reported that lactic acid, a major product of bacterial metabolism in the lower small intestine of humans, and porcine bile further stimulated encystation at pH 7.8 (Gillin *et al.*, 1989).

However, in a recent study, Lujan *et al.* (1996) demonstrated that cyst formation is induced *in vitro* by growing trophozoites in a lipoprotein-deficient medium. The authors believe that trophozoites have an affinity for the jejunum, suggesting that they require a high concentration of nutrients, including those that *Giardia* cannot synthesize such as exogenous lipid. Trophozoites, as they travel down the intestine, reach a lipid-poor environment, which could trigger their differentiation into cysts (Lujan *et al.*, 1996). However, Lujan *et al.* (1996) have shown a secondary role for bile salts and alkaline pH in *Giardia* encystation. They demonstrated that high concentrations of bile salts prevented the uptake of cholesterol and thus, favored encystation.

From these observations, two schools of thoughts have emerged. Gillin *et al.* (1989) proposed that the stimulus for encystation *in vitro* was the slightly alkaline, bile rich milieu of the intestinal lumen while, Lujan *et al.* (1996) argued that the stimulus was a starvation of cholesterol.

b) Formation of the *Giardia* cyst wall

Reiner *et al.* (1989) were the first authors to report the appearance of novel large secretory vesicles, which are called encystation specific vesicles (ESV), in trophozoites undergoing encystation. Using polyclonal antibodies (Ab), it was demonstrated that cyst antigens (Ag) were transported to the nascent cyst wall by these vesicles (Reiner *et al.*, 1989). Soon after, it was shown that early in encystation, cyst Ag are localized in simple Golgi membrane stacks and concentrated within enlarged Golgi cisternae which appear to be precursors of ESV (Reiner *et al.*, 1990). The ESV would transport the cyst Ag to the plasma membrane and release them to the cyst wall by exocytosis. Reiner *et al.* (1990) have also shown that upon completion of the cyst structure, the number of ESVs decreases on the surface of trophozoite to become absent after completion of the cyst wall. Faubert *et al.* (1991) reported that ESV, which appear on the surface membrane of the trophozoite as early as 6 hours in the encystation medium, are the earliest morphologic change observed in encystation. It was also found that trophozoites lose their ability to attach to the culture vessel wall once ESV appear (Faubert *et al.*, 1991). Using immunostaining methods and antibodies specific to cyst wall Ag, Erlandsen *et al.*(1990) demonstrated the existence of cyst wall filaments on the surface of encysting trophozoites. By using the monoclonal antibody (mAb) 8C5.C11, which recognizes cyst Ag, several researchers demonstrated that cyst wall antigens were located in the membrane-bound ESVs (Campbell and Faubert 1994a; McCafferey *et al.*, 1994).

Using immunogold labeling and high resolution field emission scanning electron microscopy (sem), Erlandsen *et al.*(1996) studied the process of encystment by localizing cyst wall specific Ag. They constructed a temporal sequence of the life cycle of *Giardia* trophozoites in vitro which has allowed them to identify an intracellular and extracellular phase in the cyst wall formation, a process which requires a total of 14-16h. *In vitro*, the intracellular formation of cyst wall precursors requires 10h while the extracellular phase requires 4-6h. The intracellular formation of the cyst wall would include the synthesis and intracellular transport of cyst wall components whereas the extracellular

phase would include the assembly and completion of the filamentous cyst wall. The studies of Erlandsen *et al.* (1996) also demonstrated that after several hours of stimulation, "caplike" structures appear on the trophozoites. These structures, are involved with filament growth, and eventually cover the entire surface of the cell body, including the flagella and ventral disc. During encystation, trophozoites changed in shape from the teardrop shape to a more spherical shape.

6. Animal Model

The ultimate objective of animal modelling in experimental medicine is to understand the disease in humans. Therefore, the animal chosen to study a particular parasitic disease should reflect sensitivity to the infection as it occurs in nature, including the pathology that will develop later without any prior experimental manipulation. In the past decade researchers have sought to develop an experimental lab model to study human giardiasis. Faubert and Belosovic (1990) reported that the ideal model to study giardiasis should be based on the following considerations: (1) sensitivity of the animal to the *G. lamblia*-type organism: (2) successful infection including colonization and multiplication of trophozoites in the small intestine: (3) formation and release of cysts in the feces: (4) infection must be transferable from humans or other hosts by oral inoculation: (5) pattern of the infection and associated pathology in the animal host should mimic the changes observed in human giardiasis: (6) animal should be inexpensive, breed under laboratory conditions and easily maintained in the laboratory. Due to the limited space, only the mouse and gerbil animal models of giardiasis will be described.

a) Gerbils

Belosevic *et al.* (1983) were the first authors to propose the Mongolian gerbil (*Meriones unguiculatus*) as an animal model for the study of human giardiasis. They showed that gerbils were highly susceptible to infection with both cysts and trophozoites of *G. lamblia* isolated from human

patients. A reproducible pattern of infection was observed, and like the infection in the human, the cyst release was intermittent throughout the infection. The gerbils were eliminating the parasites from the intestine within between six to seven weeks. During the infection, similar patterns of trophozoite distribution in the small intestine were seen in gerbils inoculated with either cysts or cultured trophozoites. Gerbils were shown to be highly susceptible to *G. lamblia* type organisms from beavers and cats and to *G. muris* organisms as well (Faubert *et al.*, 1983). Gerbils have been useful in studying the immune response and pathology to *G. lamblia* infections. For example, to study acquired immunity (Belosevic *et al.*, 1983; Lewis *et al.*, 1987); the antigenicity of different *G. lamblia* human isolates (Nash, 1989), the disaccharidase activity (Mohammed and Faubert, 1995; Belosevic *et al.*, 1989); the pathophysiology of small intestinal malabsorption (Buret *et al.*, 1991,1992) and the pathological effect of infections induced by cysts collected from human giardiasis patients (Wang *et al.*, 1986). Moreover, gerbils inoculated with *G. lamblia* isolated from an infected person, developed an infection similar to the human giardiasis (Belosevic *et al.*, 1983; Wang *et al.*, 1986). Since the the infection found in Mongolian gerbils closely mimics the infection observed in humans, it is a useful model for studying human giardiasis in the laboratory.

b) Mice

Roberts-Thompson *et al.* (1976) reported for the first time the successful infection of mice in the laboratory with *G. muris*, a natural parasite of mice. This model has provided a powerful tool to study the immune effector mechanisms that occur during *Giardia* infections. The mouse has been chosen over other animal models for a variety of reasons: first, adult mice are infected with their natural parasite; second, many reagents and assays to study the immune response in mice are available; and third, the existence of immunologically well defined inbred strains of mice are very useful (Faubert, 2000). For example, B and T-cell deficient and mast-cell deficient mice have been useful to understand the mechanisms of the immune response to the infection (Snider *et al.*, 1988;

Robert-Thomson and Mitchell, 1978; Erlich *et al.*, 1983). Among the several different strains of mice that exist some appear to be susceptible to *Giardia* infection while others have shown resistance (Belosevic *et al.*, 1984). For instance, in susceptible A/J and C3H/HeJ mice the pattern of the infection is characterized by a short latent period, a high cyst output during the acute phase and a relatively long period of elimination. In contrast, resistant B10.A and DBA/2 had a prolonged latent phase, a lower cyst output during the acute phase and a rapid resolution of the infection. On the other hand, the giardiacidal activity of spleen, mesenteric lymph nodes (MLN) and peritoneal lymphoid cells from susceptible or resistant mice showed no difference (Belosevic and Faubert, 1986). Also, no apparent relationships were found between the capacity of susceptible and resistant mice to mount cell mediated effector responses and their ability to control the infection with *G. muris* (Belosevic and Faubert, 1986). Unfortunately, the mouse model is not useful to study *G. lamblia* since this parasite completes its life cycle in immunologically immature mice only.

7. Antigens of *G. lamblia*

Knowledge of the antigenic composition of the parasite and the role that these antigens play in the immune response during infection is important for the understanding of immunity. However, elucidation of the *Giardia* antigens involved in the immune response has been difficult for the following reasons. First, protective immunity is only partial; second, some infections are asymptomatic; third, antigenic variation occurs within and among isolates; and last, different techniques have been used to characterize antigens which makes comparison of results from different laboratories difficult. One should be careful in assuming that similar molecular weight antigens reported by different laboratories are the same antigen. An extensive characterization of the epitopes must be done.

a) Trophozoites

Several trophozoite antigens of molecular mass varying between 24 and 225 KD have been detected by immunoprecipitation and immunobloting techniques using polyclonal or MAb. Many of these antigens are shared by different isolates. An 82 KD surface glycoprotein antigen common to four different isolates was identified by Einfield and Stibbs (1984). This 82 KD has been detected in the sera of 29 infected patients (Ortega-Pierres *et al.*, 1988). In a different study, Kumkum *et al.* (1988a) revealed that an 82 KD surface antigen is present in three human isolates. Human patients with non persistent giardiasis developed Immunoglobulin (Ig) M antibodies to the 82 KD antigen which coincided with the resolution of the infection (Kumkum *et al.*, 1988b). A band corresponding to a 57 KD antigen was recognized by IgG antibodies in the sera of all *Giardia* patients but not in the sera of control patients. Upcroft *et al.* (1988) identified a 32 KD antigen which was common to 13 isolates of *G. lamblia*. It was also found that human anti-*G. lamblia* sera recognize an 88 KD protein present on the surface of axenically cultured trophozoites (Edson *et al.*, 1896). Other antigens of *G. lamblia* have been identified but their importance in the immune response is not defined. These include a 49 KD surface glycoprotein (Meng *et al.*, 1990) and 55 and 53 KD tubulin molecules (Torian *et al.*, 1984). Recently, antigens of 170, 105, 92, 66, 32, 29 and 14 KD in saliva samples from *Giardia* infected patients showed a response against the trophozoite membrane fraction (Rosales-Borjas *et al.*, 1998). These antigens could be important in protection or diagnosis of *G. lamblia* infections. Studies have been done to clone the genes encoding those antigens. Gillin *et al.* (1990) have cloned a gene that encodes a 72.5 KD protein. Sequence analysis of this gene revealed a single open reading frame specifying a hydrophilic, cysteine-rich (11.8%) protein with an amino-terminal signal peptide and a postulated hydrophobic membrane spanning anchor region near the carboxyl terminus. Adam *et al.* (1992) have cloned a gene which expresses a cysteine-rich 170 KD surface protein (CRP170) that undergoes antigenic variation. Mowatt *et al.* (1991) have also cloned a gene which encodes a variant surface protein, VSP1267.

b) Cysts

Reiner *et al.* (1989) were the first to characterize cyst-specific antigens of *G. lamblia*. Using a rabbit polyclonal anti-cyst serum, they have identified a heterodisperse group of low-molecular mass antigens (~21 to 39 KD) appearing early during *in vitro* encystation. These authors have also identified antigens of ~66, 78, 92 and 103 KD which appear later in encystation (~24 h). Using cyst specific serum and a mAb, Erlandsen and colleagues (1990) identified cyst wall antigens of 29, 75, 88 and 102 KD. Ward *et al.* (1990), recognized four antigens of 29, 36, 39 and 45 KD using a mAb raised against *Giardia* cysts. In 1992, Reiner and Gillin have demonstrated that secretory and serum IgA, IgM and IgG antibodies from giardiasis patients recognize cyst antigens of 66, 78, 94, 103 and 120 KD. Using two mAb, which recognize protein cyst antigens, Cambell and Faubert (1994a) revealed that mAb 8C5.C11 recognized cyst antigens of 26, 28, 42 and 46 KD while mAb 5A4.G6 reacted with a 38 KD protein. Using Western blot, the two mAb were shown to be specific to the cyst structure. Mowatt *et al.* (1995) were the first to clone a gene which encodes a protein component of the cyst wall and ESV. The sequence of the cloned gene identifies the expression of an acidic, leucine-rich cyst wall protein (CWP) 1 of 26 KD. Lujan *et al.* (1995) cloned the second gene which encodes a 39 KD cyst wall protein, CWP2, by using a mAb against cyst wall antigens.

8. *CWP1* and *CWP2*

CWP1 and *CWP2* are acidic and leucine rich proteins with molecular weight of 26 and 39 KD respectively (Mowatt *et al.*, 1995; Lujan *et al.*, 1995).

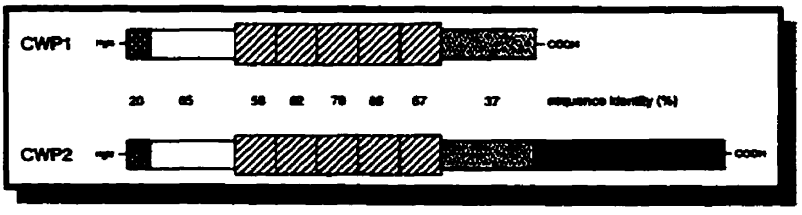

Figure 3. Schematic depiction of *CWP1* and *CWP2* based on their deduced amino sequences. The two closely related secretory proteins contain leucine-rich repeats (hatched boxes) but are distinguished by a strongly basic carboxyl-terminal tail (in black). (modified) (From Lujan *et al.*, 1995)

They are the only defined protein constituents of the *Giardia* cyst wall and are closely related in primary structure. The two *CWP*s possess a hydrophobic amino terminal signal peptide which precedes a five tandemly arrayed 24-residue leucine rich repeats (LRR) followed in turn by a cysteine-rich region (Fig. 3) (Lujan *et al.*, 1995). However, unlike *CWP1*, *CWP2* has a 121-residue carboxyl-terminal extension of 13KD that is rich in basic amino acids (Fig. 3). Interestingly, both proteins show positional amino acid sequence identity of 61% in the 241-residue overlap of the two proteins (Fig. 3). This largely takes place in the LRR region and the domain immediately before. The expression of both *CWP1* and *CWP2* is upregulated during encystation *in vitro* (Mowatt *et al.*, 1995; Lujan *et al.*, 1995). The levels of the transcripts from the two *CWP*s genes increased to a maximum of 140 times that observed in non-encysting trophozoites. In addition, both *CWP*s are found within the ESV of encysting trophozoites and in the cyst wall of mature cysts (Lujan *et al.*, 1997). It was also found that both proteins are expressed coordinately, forming a stable complex through disulfide bonds within five minutes of their synthesis.

It seems evident that *CWP1* and *CWP2* are deeply involved in the phenomenon of encystation of *Giardia* but many questions remain unanswered. For example, how does the integration of proteins occur in a solid structure, like the cyst? The presence of the amino terminal signal peptide found in *CWP1* and *CWP2* is an indication that these two proteins will be secreted (Lujan *et al.*, 1995). Lujan *et al.* (1997) proposed that aggregation of a *CWP1-CWP2* receptor complex could result in ESV budding. They also advanced that the formation of ESV could be a direct consequence of the synthesis of the *CWP*s, especially *CWP2*. In purified ESV, *CWP2* was mainly found as a 39 KD protein whereas only a 26 KD fragment could be found in purified cyst wall. These results would suggest that a proteolytic processing of the 13 KD terminal extension of *CWP2* occurred during its incorporation into the cyst wall.

The accumulation of both *CWP1* and *CWP2* in a disulfide-linked form encysting trophozoites

and its five LRR, suggests that these proteins are a constituent of the fibrillar component of the cyst wall (Lujan *et al.*, 1995). The LRR regions may serve as a flexible domain that facilitates protein-protein interactions (Kobe and Deisenhafer, 1994). The LRR consensus sequences of the *Giardia CWP*s resemble those found in the extracellular domain of plant trans-membrane and extracellular matrix proteins (Ohsumi *et al.*, 1993; Kresse *et al.*, 1994).

It was found by Lujan and Nash (unpublished data) that MAb 8C5.C11 binds recombinant *CWP2* in Western blot assays. Interestingly, Campbell and Faubert (1994) showed that *in vitro* encystation can be interrupted by the addition of mAb 8C5.C11. These findings indicate that *CWP2* could be a potential candidate Ag for a vaccine against cyst formation. Another reason to use *CWP2* is that it appears to be a stable invariant protein component required for cyst wall formation. This may lead to innovative methods for interfering with production of an intact viable cyst wall, and therefore, regulation of cyst release from the host.

9. Mucosal Immune System

The mucosal immune system is made up of lymphoid tissue distributed throughout the mucosal area of the body. It is the primary mediator of specific immunity at mucosal surfaces. Thus, it plays a role in maintaining homeostasis and in defending the host against overt and opportunistic pathogens (McGhee *et al.*, 1992). The mucous membrane lining the digestive, respiratory and urogenital system, which have a combine surface area of 400m^2 (Brown, 1996), are the major sites of entry for most

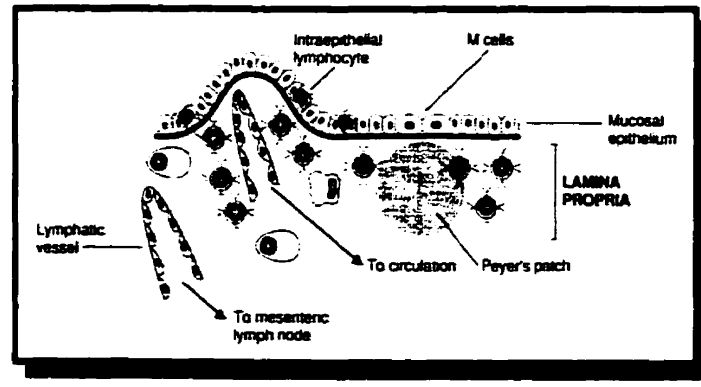

Figure 4. Cellular component of the mucosal immune system. (From Abbas *et al.*, 1994)

pathogens. At the intestinal mucosal sites, epithelial cells, secretory (S)-IgA and gut-associated lymphoid tissues (GALT) are playing a major role. The GALT includes the intraepithelial lymphocytes (IEL), the lamina propria lymphocytes (LPL), Peyer's patches (PP) and mesenteric lymph nodes (MLN) (Fig. 4). One of the distinguishing feature of this immunity is the preferential production, transport and secretion of IgA at all mucosal surfaces which is done by mechanisms remarkably distinct from those involved in systematic antibody responses (McGhee *et al.*, 1989).

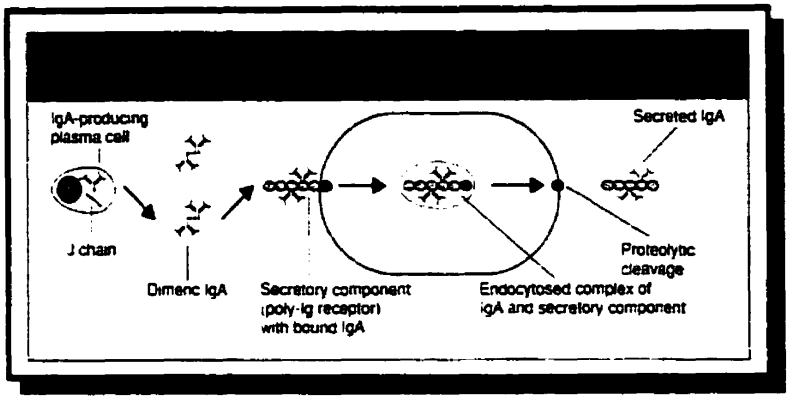

Figure 5. Mechanisms of IgA transport and secretion in mucosal epithelia. IgA is produced by plasma cells in the lamina propria and binds to secretory component. The complex is actively transported through the epithelial cell, and the bound IgA is released by proteolytic cleavage (From Abbas, *et al.*, 1994)

IgA exists in 2 forms: the first one is found in serum and the second one, S-IgA, is found in all mucosal sites. Serum IgA exists as a monomer whereas the S-IgA consists of a dimer, a J chain polypeptide and a polypeptide chain called secretory component (SC). In the gastrointestinal tract, the process of S-IgA is initiated by the entry of protein Ag into the PP through "microfold" cells (M cells) (Neutra *et al.*, 1996). After crossing the epithelial barrier, Ags are internalized by antigen-presenting cells (APC). APC are defined as cells that handle and present Ag to T lymphocytes and primarily include dendritic cells and B cells (Reyes *et al.*, 1997). Upon activation, B cells proliferate and switch to IgA committed cells. The IgA cells leave the GALT and migrate to distant mucosal sites where they undergo terminal differentiation to IgA-producing plasma cells (Frey and Neutra, 1997). Plasma cells secrete IgA antibodies which forms dimers through the polypeptide chain J (Fig. 5). The polymeric IgA is then taken up by the polymeric

immunoglobulin receptor found on the basolateral surfaces of epithelial cells lining mucous (Mostov, 1994). The complexes will then be transported through the epithelial layer in endocytotic vesicles. The polymeric immunoglobulin receptor is cleaved and IgA is secreted wrapped within a residual secretory component into the external secretion. The structure of S-IgA affords the molecule resistance to most proteases and increases its functional affinity for corresponding Ags (Bouvet and Fischetti, 1999). Molecular and cellular aspects of regulatory T cells, IgA committed B cells and APC in PP have shown that CD4 T cells and their derived cytokines regulate the S-IgA response (McGhee *et al.*, 1992).

10. B-Cell Mediated Immunity in Giardiasis

The importance of humoral immunity in human giardiasis was first suggested by Brown *et al.*(1972) who observed that patients with generalized hypoglobulinemia were frequently infected with severe and prolonged symptomatic giardiasis. In immuno-competent patients, serum antibodies (Ab) have been reported (Janoff *et al.*, 1988; Nash *et al.*, 1987; Smith *et al.*, 1981). Hill *et al.* (1984) found that sera from infected patients have Ab that could kill up to 98% of the trophozoites *in vitro*. The attachment of *G. lamblia* to intestinal epithelial cells can be inhibited by trophozoites specific Ab (Inge *et al.*, 1988).

a) IgA and S-IgA

IgA is the predominant and possibly the only effector Ab active to control ths intestinal infectious agent. In human, *Giardia* specific IgA Ab have been reported in the serum (Roberts and Anders, 1981; Janoff *et al.*, 1990) and in a secretory form in the intestine (Nash *et al.*, 1987), in maternal milk (Miotti,1985; Nayak *et al.*, 1987) and in saliva (Speelm and Ljungstrom, 1986). Deficiencies in both serum IgA and S-IgA have been associated with chronic giardiasis in humans (Susano *et al.*, 1993).

Several studies have reported the presence of anti-*Giardia* IgA in serum from patients with giardiasis. although recent studies suggest that only about 30% of patients with active infection have detectable anti-*Giardia* IgA Ab in the serum (Goka *et al.*. 1989; Char *et al.*. 1991). Serum anti-*Giardia* IgA Ab levels were also found to be low in studies on giardiasis in children (Shetty *et al.*. 1992). On the other hand. Perez *et al.* (1994) found that serum IgA Ab levels were higher in asymptomatic patients compared to symptomatic patient. In addition. high levels of serum-specific IgA has been associated with high exposure to *Giardia* (Janoff *et al.*. 1990). A substantial IgA Ab response in the serum and gut secretions of *G. muris*-resistant mice was found (Snider and Underdown. 1986). On the other hand. Daniels and Belosevic (1994). using *G. muris* infected mice. suggested the absence of relation between the degree of protective immunity and the presence of serum-specific IgA.

By using immunofluorescence. Briaud *et al.* (1981) have shown the presence of S-IgA on the surface of *G. lamblia* trophozoites in human jejunal biopsies. It was observed that s-IgA was bound to the surface of *G. lamblia* trophozoites collected from the intestine of infected rats (Sharma and Mayrhofer. 1988). Loftness *et al.* (1984) also found S-IgA on the flagella and dorsal and ventral surfaces including the adhesive disc of *G. lamblia* trophozoites found in infected rats. In mice. both S-IgA and IgA have been demonstrated on the surface of *G.muris* trophozoites (Heyworth *et al.*. 1988) and eradication of infection was closely dependant on increasing concentrations of specific S-IgA in intestinal fluid (Snider and Underdown. 1986; Heyworth *et al.*. 1988). Snider and Underdown (1986). demonstrated in Balb/c and C57BL/6 *G. muris* infected mice. that S-IgA is the only Ab in the gut which can be found throughout the course of infection. Using nude mice infected with *G. muris*. it was reported that the lack of intestinal anti-*Giardia* trophozoite S-IgA Ab was responsible for the chronic type of infection (Heyworth *et al.*. 1988) . It was also reported that the highest titers of *Giardia* specific S-IgA in the duodenal fluid of asymptomatic carriers compared to

the acute or chronic diarrhoea group (Randhawa *et al.*, 1992). These findings suggest that S-IgA may play a role in the resolution of human giardiasis. It was also found that mothers with giardiasis had S-IgA in their milk (Nayak *et al.*, 1987). The infants breast fed by mothers with giardiasis had a lower incidence of infection than infants breastfed from mothers without giardiasis suggesting a protective role for passively acquired antibody in humans, specifically S-IgA.

b) IgM and IgG

Non-specific IgM levels are slightly elevated in patients with recently acquired giardiasis (Jokipii and Jokipii, 1982). Serum anti-*G. lamblia* IgM Ab have been detected in all infected persons (Nash *et al.*, 1987). IgM Ab titers increase in early infection, but return to normal level three weeks after infection (Goka *et al.*, 1986; Nash *et al.*, 1987). Kumkum *et al.* (1988a) found that IgM Ab levels were higher in patients with acute/non-persistent giardiasis as opposed to patients with persistent giardiasis. The role of IgM Ab in killing trophozoites was confirmed when sera from infected humans lysed trophozoites (Deguchi *et al.*, 1987). Butscher and Faubert (1988) confirmed these results when they reported that IgM mAb raised against *G. muris* trophozoites were capable of killing trophozoites *in vitro* in the presence of complement. When they passively immunized mice with the mAbs, the numbers of trophozoite was significantly reduced in a challenge infection.

There is some evidence that IgG Ab may help in the clearance of *G. muris* infections in mice. Heyworth (1986), using immunofluorescence microscopy, found that parasite-specific IgG and IgA Ab bind to *G. muris* trophozoite colonizing the small intestine. Snider *et al.* (1985,1988) have found that specific anti-*Giardia* Ab have been detected in sera of mice during experimental infection with *G. muris*. They detected IgG and IgA Ab two to three weeks after initiation of infection but no measurable IgM Ab was detected. It was found that serum anti-*G. muris* IgG Ab appeared earlier in resistant mice as opposed to susceptible mice (Anders *et al.*, 1982).

Several studies have demonstrated that IgG isotype support the lytic effect of complement

on *Giardia* cells. It has been shown that specific IgG1 (Nash *et al.*, 1988) and IgG3 (Heyworth, 1992) mAb can kill trophozoites *in vitro* in the presence of complement. However, IgG1 mAbs were able to inhibit the growth of trophozoites *in vitro* in the absence of complement. Belosevic *et al.* (1994) observed a significant decrease in the trophozoite burden and cyst release when they administered an anti-*G. muris* IgG1 mAb added to exogeneous complement intraduodenally to *G.muris* infected mice. When Campbell and Faubert (1994) added the IgG1 mAb 8C5.C11, which recognize cyst Ag, with complement to an encysting culture, they observed a reduction of more than 60% in the numbers of water resistant cysts.

In humans, anti-*Giardia* IgG Ab are present in sera (Smith *et al.*, 1981; Roberts-Thomson and Anders, 1981) and in mother's milk (Miotti *et al.*, 1985). IgG Ab can be detected in more than 80% of patients with symptomatic giardiasis (Goka *et al.*, 1986). The level of IgG Ab remains elevated for month or even years after primary infection. Asymptomatic individuals in endemic area have been found to have anti-*Giardia* IgG in their serum indicating previous exposure to the parasite (Gilman *et al.*, 1985). However, a relationship between IgG and protection has not yet been demonstrated.

11. Cell Mediated Immunity in Giardiasis

Evidence suggests that cell mediated immunity is involved in giardiasis. Several reports show that there is a large increase in the number of intraepithelial lymphocytes during giardiasis (Rosekrans *et al.*, 1981; Ferguson *et al.*, 1976; Wright and Tomkins, 1977). In BALB/c mice infected with *G. muris*, lymphocytes numbers in Peyer's patches (PP) doubled during the course of experimental infection but returned to normal levels when the infection was resolved (Carlson *et al.*, 1986). T cells play a major role in the immune response to *Giardia* in mice. There are two major populations of T cells: T helper (Th) cells and T cytotoxic (Tc) cells. The Th cells and the Tc

cells can be distinguished by the presence of one of two membrane glycoproteins, either CD4 or CD8. T cells displaying CD4 glycoproteins generally function as Th cells, whereas those displaying CD8 glycoproteins generally function as Tc cells. The inability of nude (athymic) mice to eliminate infection with *G. muris* points to the importance of T-cell dependant immune mechanisms in the resolution of the infection (Roberts-Thompson and Mitchell, 1978; Stevens *et al.*, 1978). Heyworth *et al.* (1985) found that nude mice infected with *G. muris* developed chronic giardiasis. Corticosteroid (Nair *et al.*, 1981) or cyclosporin A (Belosevic *et al.* 1986) treated mice infected with *G.muris* had increased numbers of parasites. Since these agents primarily suppress T cell functions, it suggest the importance of T-cell immunity in the clearance of giardiasis. Heyworth *et al.*(1987) used BALB/c mice depleted of CD4 or CD8 cells to determine which T cell-subset was important in the resolution of the infection. Mice depleted of CD4 cells demonstrated defective clearing of *Giardia* parasite whereas those depleted of CD8 cells resolved their infection normally. These results suggests that Th cells play a more important role than Tc cells in the clearing of giardiasis. Subsequently, it was shown that mice depleted of Th cells have an impaired IgA response to *G. muris* (Heyworth, 1989). This would further suggest that Th cells are required to stimulate an IgA response, likely involved in the resolution of giardiasis.

Mast cells play a role in helping to clear *Giardia* infections since mice deficient in those cells experience prolonged experimental infection (Erlich *et al.*, 1983). However, natural killer (NK) cells do not seem to aid in expulsion of the parasite considering that beige mice (NK cell deficient) clear infection of *G. muris* as rapidly as immunocompetent C57BL/6J mice (Heyworth *et al.*, 1986).

Intestinal macrophages from mice infected with *G.muris* have been shown to phagocytize trophozoites (Owen *et al.*, 1981). Evidence has reported that immune serum enhanced the intestinal or PP macrophage activity *in vitro* against *Giardia* trophozoites (Radulescu and Meyer, 1981; Kaplan *et al.*, 1985; Hill and Pohl, 1990). This indicates that an antibody-dependant cell-mediated

cytotoxicity (ADCC) process is involved in the phagocytosis of trophozoites by macrophages. However, no difference in macrophage phagocytosis has been found between mice susceptible or resistant to *G. muris* infection (Owen *et al.*, 1981; Heyworth *et al.*, 1985; Belosevic and Faubert, 1986). In addition to a possible role as being effector cells for resolution of the parasite, macrophages probably act as APC for CD4 lymphocytes and/or participate in antibody-mediated killing of the trophozoites (Adam *et al.*, 1991).

12. Cytokines

Cytokines, which are proteins of low molecular weight, are critical in the regulation of immune responses. They regulate the intensity and duration of the immune response by stimulating or inhibiting the activation, proliferation, and/or differentiation of various cells and by regulating their secretion of antibodies or other cytokines. Cytokines can be pleiotropic (have different biological effects on different target cells), redundant (have similar effects), synergistic (have combined effects) and antagonist (have inhibiting effect on the action of another cytokine), all of which help them to coordinately regulate cellular activity. Although a variety of cells can secrete cytokines, the two principal producers are the Th cells and macrophages. *In vitro* studies of Th cells clones first indicated the possibility of two types of Th cells on the basis of the patterns of cytokines secreted (Mosmann *et al.*, 1986; Mosmann and Coffman, 1989). Th cells were classified as Th1 cells, which control the cell mediated immune responses and produce mainly interleukin (IL)-2 and interferon gamma (IFN-γ) and Th2 cells, which control the humoral immune responses and produce mainly IL-4 and IL-5. Both types of Th cells have CD4+ markers on their surface.

During *in vitro* studies of spleen cells of mice, it was found that both Th1 and Th2 cells originate from the same precursors which is Th0 cells (Gajewski *et al.*, 1989). Depending on the cytokines present when the stimulation takes place, Th0 cells will differentiate into either Th1 or Th2

(Mosmann *et al.*, 1986; Fiorentino *et al.*, 1989). IFN-γ is critical in the development of Th0 into Th1 cells and inhibits the development of Th2 cells although it has no effect on IL-4 production (Noble *et al.*, 1993; Gajewski and Fitch, 1988). On the other hand, IL-4 is essential in the development of Th2 cells subset (Paul and Seder, 1994). Moreover, IL-4 alone is able to maintain the proliferation of Th2 cells (Noble *et al.*, 1993).

a) Cytokines and immune responses

IFN-γ is the primary macrophage-activating factor, thus, it is a potent cytokine in cell-mediated immunity. An increased production of reactive oxygen intermediate will result from the activation of macrophages by IFN-γ. This action represents the major mechanism used in the killing of intracellular parasites (Abbas *et al.*, 1991). IFN-γ and IL-4 have antagonist action upon their effect on class II MHC expression by APCs (Mond *et al.*, 1986; Gajewski *et al.*, 1989). IFN-γ increases MHC class II expression, enhancing Ag presentation of macrophages and monocytes. The Ag presentation will favour the development of a Th1 immune response. However, IL-4 will increase MHC class II expression of B cells acting as APC, which favours a Th2 immune response. IFN-γ can also increase MHC class I expression on various cells (Kuby, 1993). IL-5 promotes growth and differentiation of eosinophils (Coffman *et al.*, 1989). The functional capacity of eosinophils is also increased by this cytokine (Abu-Ghazaleh *et al.*, 1992).

Other studies have shown that IFN-γ can also regulate the humoral immune responses. It was found that IFN-γ enhances the activation of IgM secreting B cells (Leibson *et al.*, 1984). In addition, IFN-γ increases the production of both IgG2 precursor (Snapper and Paul, 1987). IFN-γ deficient mice were immunized orally with tetanus toxoid (TT) as Ag and cholera toxin (CT) as mucosal adjuvant to examine the importance of IFN-γ in the development of vaccine induced mucosal S-IgA and serum IgG response (Vancott *et al.*, 1997). Strong systemic IgG and mucosal S-IgA antibody responses were elicited which suggest that IFN-γ was not essential to raise a

mucosal S-IgA response. IL-4 and IL-5 are the two major cytokines involved in the regulation of the humoral immune response. High levels of IL-4 trigger a switch of Ab from S-IgM to IgG4 (Kotowicz and Callard, 1993) while low levels of IL-4 triggers a switch from S-IgM to IgG1,2,3 and IgA. In addition, IL-4 inhibits the production of IgG2a, which IFN-γ upregulates. Mice immunized orally with TT and CT had significant increase in TT-specific IgG1 and IgE, both produced by Th2 cell derived IL-4 (Vancott *et al.*, 1997). IL-4 knockout mice also immunized orally with TT and CT demonstrated low Th2-type responses and are unable to generate mucosal S-IgA Ab to TT. This study demonstrates that IL-4 was essential to induce the production of TT specific S-IgA (Kopf *et al.*, 1993; Okahashi *et al.*, 1996). On the other hand, IL-5 is one of the most potent cytokine in mediating differentiation of IgA-B cells to become IgA-producing plasma cells (Sonoda *et al.*, 1992).

b) Cytokines and *Giardia*

Venkatesan *et al.* (1996) compared the MLN cytokine production in a strain of mice susceptible to *G. muris* and a strain of mice resistant to the parasite. When cells harvested from the MLN were stimulated with concanavalin A, IFN-γ and IL-5 were secreted by cells from resistant strains, but only IL-5 was secreted by cells from susceptible strains. This study suggests a role for IFN-γ in the control of the *G. muris* infection. Djamiatun and Faubert (1998) were the first to report on the cytokines produced by CD4+ T cells in response to *Giardia* antigenic stimulation. They used *Giardia* trophozoite proteins to challenge PP and spleen cells of infected mice and found that neither IL-4, IL-5 and IFN-γ were detected. However, when they stimulated the cells with concanavalin A, all the cytokines were detected. The detection of IL-4 and IL-5 confirms the role played by Ab of the IgA isotype in the control of giardiasis while the detection of IFN-γ confirms the role played by the Th2 mediated immune response in the resolution of the infection. In addition, *in vitro* studies have shown that IFN-γ and macrophages played a central role in the killing of trophozoites (Belosevic and Daniels, 1992).

13. Adjuvants

Adjuvants (from the latin *adjuvare*. to help) are substances that nonspecifically enhance or potentiate the immune response to an Ag. Adjuvants are frequently used to stimulate the immune response when an antigen presents low immunogenicity or when only small amounts of an antigen are available. From the early 1920's adjuvants have been used to increase the efficacy of vaccines (Cox and Coulter. 1997). Different modes of action of some commonly used adjuvants have been postulated. These modes of action include prolonging antigen persistence. inducing granuloma formation. stimulating lymphocytes nonspecifically and enhancing co-stimulatory signal. Many adjuvants are also known to have an immunomodulation effect, which refers to their ability to modify the cytokine network. Some adjuvants may cause a partial swing to either a Th1 or a Th2 immune response.

a) Cholera toxin

Cholera toxin (CT) is the primary exotoxin produced by *Vibrio cholerae* bacteria and is responsible for the symptoms associated with cholera disease. It is a potent mucosal adjuvants in some animal models and is part of a group of adjuvants called bacterial toxins. The adjuvant action of bacterial toxins is not understood and may involve molecular targeting via the GM1 ganglioside receptor or stimulation of endogenous adenylate cyclase activity. CT consists of five binding (B) subunits assembled into a ring into which a toxic-active (A) subunit is inserted. Most mammalian cell types carry specific CT receptors in the form of GM1 ganglioside on their cell. The B subunit will bind the toxin with affinity to those receptors and then the A subunit will be translocated into the cell. The receptor will respond with cyclic AMP formation to the ADP-ribosylating intracellular enzyme activity of the A subunit of CT. Hence, CT can modulate the function of a variety of cells, not only those directly involved in the cholera diarrheal secretory process but also cells of the immune system including lymphocytes and APC.

In recent years, CT has attracted much interest because of its ability to act as a potent mucosal adjuvant which stimulates a strong immune response (mucosal and systemic) to unrelated antigens following oral immunizations. CT has been found to overcome a state called oral tolerance when used as a mucosal adjuvant (Lycke and Holmgren, 1986; Elson and Ealding, 1984). The majority of proteins, being weekly immunogenic, require large immunization doses and often producing oral tolerance (when given orally) rather than stimulation (Chiller and Alosbrook, 1986). CT has different immunomodulatory functions. For example, it stimulates IgA responses to unrelated protein antigens when co-administered orally (Elson and Ealding, 1984). This confirms that CT prioritize a swing to a Th2 immune responses. It also enhances antigen presentation by increasing IL-1 and IL-6 (Bromander *et al.*, 1995) and increases the frequence of primed Ag specific to T lymphocytes (Hornquist and Lycke, 1995). CT also augments gut permeability to luminal antigens (Bromander *et al.*, 1995) and promotes the development of a long term immunological memory in the intestine (Vajdy and Lycke, 1992; Lycke and Holmgren, 1989; Elson and Ealding, 1984). Oral immunization with *Trichinella spiralis* antigenic preparations, mixed with CT as the adjuvant, resulted in reduction in worm size, in fecundity and in the mean of total muscle larvae (DeVos and Dick, 1993). Such reductions were not observed in mice fed *T. spiralis* Ag without CT or fed CT alone. Also, oral vaccinations with *Trichuris muris* adult worm homogenate antigen with CT as the adjuvant resulted in high levels of protection against subsequent infection (Robinson *et al.*, 1995).

MANUSCRIPT

STUDIES ON THE IMMUNE RESPONSE OF BALB/c MICE IMMUNIZED ORALLY WITH *GIARDIA LAMBLIA* CYST WALL PROTEIN

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(To be submitted for publication)

ABSTRACT

In this study, we determined the immunogenicity of *CWP2* and its potential as a vaccine candidate against giardiasis. *CWP2* was expressed as a recombinant protein with an hexa-histidine affinity tag and was isolated from inclusion bodies. When BALB/c mice were immunized with *CWP2*, a specific IgA was detected in the feces. When mice were immunized with *CWP2* + cholera toxin, as an adjuvant, IgA in the feces, and IgA, IgG1, and IgG2a in the serum, all specific to *CWP2*, were detected. Also, CD-1 mice were infected with *G. muris* and presence of specific IgA antibodies to *CWP2* were detected in the feces. This result indicated that *CWP2* was recognized by the immune system in a natural infection. IL-4 and IL-5 were released from Peyer's patches (PP) and mesenteric lymph nodes (MLN) cells when stimulated with concanavalin A. In spleen cells, IFN-γ, IL-4, and IL-5 were released when stimulated with concanavalin A. However, in PP, MLN and spleen cells, the levels of cytokines were barely detectable when stimulated with *CWP2*. The presence of IgG2a (Th1), IgA and IgG1 (Th2) as the production of IFN-γ (Th1), IL-4 and IL-5 (Th2) confirmed that *CWP2*, when presented orally to mice, stimulates both a Th1 and Th2 type immune response, locally and systemically.

INTRODUCTION

Giardia lamblia is an intestinal protozoan parasite of humans and other vertebrates and has a worldwide distribution. *Giardia* infections can be asymptomatic, but oftentimes present acute and chronic debilitating disease associated with diarrhea and malabsorption (Adam, 1991). Transmission may occur by personal contact with infected people, by drinking contaminated water or by eating food contaminated with cysts. Because of the ability of *Giardia* cysts to survive in freshwater for long periods (Faubert *et al.*, 1986), this parasite is known to be the most common cause of epidemic waterbone diarrheal disease (Adam, 1991). Outbreaks of giardiasis have been associated with inadequate water treatment. Wallis *et al.* (1996) have recently studied the prevalence of *Giardia* cysts isolated in running surface water in Canada. Cysts were found in 73% of raw sewage samples, in 21% of surface water samples and in 18.2% of chlorinated filtered water samples. There is compelling evidence that wild and domestic animals are playing a role in the spreading of the infection in the environment. For example, beavers were implicated in an outbreak in British Colombia in 1986 (Moorehead *et al.*, 1990). Muskrats were reported to be responsible for contaminating surface water in Germany (Karanis *et al.*, 1996). Cats and dogs have also been shown to carry *Giardia* (Leib *et al.*, 1999) and could become a source of infection for their owners. A study that looked at six different farms in southern Alberta found that the overall infection rate for *Giardia* in sheep and cattle was 10.4% (Buret *et al.*, 1990). In a more recent study, Ruest *et al.* (1998) looked at the prevalence of *Giardia* in dairy farms in Quebec. They found that 45.7% of the cattle farm samples were found to be positive for *Giardia*. A survey of 66 surface water treatment plants in 14 states and in one Canadian province showed that surface water receiving agricultural effluent had

a higher density of *Giardia* cysts than protected water sheds (LeChevalier *et al.*, 1991). This suggests that farm animals play a major role in spreading the infection throughout the environment.

Giardia has a simple, direct life cycle, which includes two life stages: the cyst and the trophozoite. Trophozoites undergo fundamental morphologic changes to survive outside the intestine of their host, in the environment, by differentiating into infective cysts. The rigid filamentous cyst wall renders cysts resistant to harsh environmental conditions, hence, participants in the successful transmission of giardiasis, and furthermore, participants in the contamination of the environment (Erlandsen *et al.*, 1990). Encystment is essential for the transmission of *Giardia*, and as such, represents a target for reducing or stopping the spread of cysts in the environment.

During *in vitro* (Reiner *et al.*, 1989) and *in vivo* (Campbell and Faubert, 1994) encystation, novel structures on the membrane surface called encystation-specific vesicles (ESV) develop. These vesicles are responsible for the transport of stage specific proteins to the nascent cyst wall (Reiner *et al.*, 1990). The proteins transported by the ESV to the cell wall appear to be essential for the construction of the cyst wall.

Cyst antigens of molecular mass ranging between 21 and 49 kilodalton (KD) were detected in human feces (Gillin *et al.*, 1987). These polypeptides were not found in the trophozoites. Polypeptides ranging from 29 to 45 KD in immunoblot and immunofluorescence were recognized by mAb raised against cyst antigens (Ward *et al.*, 1990). The polypeptides appeared within eight hours of exposure of the trophozoites to encystation medium (Campbell and Faubert,1994; Ward *et al.*, 1990). These authors concluded that the molecules appearing early during encystation represent potential targets for strategies directed at inhibiting the process of encystation.

Two genes expressing protein components of the cyst wall have been cloned. One of the

cloned genes expresses an acidic leucine rich 26KD cyst wall protein (*CWP*) 1 (Mowatt *et al.*, 1995). The second cloned gene also encodes an acidic leucine rich cyst wall protein *CWP2*, but of 39KD (Lujan *et al.*, 1995). The two *CWP*s possess a hydrophobic amino terminal signal peptide which precedes a five tandemly arrayed 24-residue leucine rich repeats (LRR), followed in turn by a cysteine-rich region (Lujan *et al.*, 1995). However, unlike *CWP1*, *CWP2* has a 121-residue carboxyl-terminal extension of 13KD that is rich in basic amino acids. Interestingly, the expression of both *CWP1* and *CWP2* is upregulated during encystation *in vitro* (Mowatt *et al.*, 1995; Lujan *et al.*, 1995). The levels of the transcripts from the two *CWP*s genes increased to a maximum of 140 times that observed in non-encysting trophozoites. In addition, before both *CWP*s are incorporated into the nascent cyst wall, they are found within the ESV of encysting trophozoites and later in the cyst wall of mature cysts (Lujan *et al.*, 1997).

Campbell and Faubert (1994) developed a monoclonal antibody (mAb) 8C5.C11 against encysting cells antigens. When 8C5 was added to encysting culture, the formation of cysts *in vitro* was reduced by 60%. A similar percentage of reduction was observed when crude extract of encysting cells was used to immunize mice orally with cholera toxin (CT) as an adjuvant. When the mice were challenged with live cysts, they released fewer cysts in their feces compared to non-immunized mice. It was shown that mAb 8C5.C11 is specific to cyst antigens and as such, does not react with trophozoite antigens (Campbell and Faubert, 1994; McCaffery *et al.*,1994). These results show that the antigens binding to mAb 8C5 are candidates for the development of a vaccine directed against the infective stage of the disease, the cyst.

There are few studies on the induction of active immunity against *G. lamblia*. Prior subcutaneous immunization followed by oral immunization of mice with a 56 KD *G. lamblia*

trophozoite protein resulted in lower loads of trophozoites in the small intestine (Vinayak *et al.*, 1992). The immunization stimulated CD4+ T cells for a short period, but returned to normal levels by day 30 post-immunization. A significant elevation in the number of IgA and IgG plasma cells were also observed. Smaller numbers of cysts excreted in the feces were observed when six-week old kittens were immunized with crude extracts of trophozoites (Olson *et al.*, 1996). *Giardia*-specific IgG and IgA antibody was detected in the serum. The mucosal anti-*Giardia* IgA antibody titer in the vaccinated kittens was also increased. The experiment was repeated in six-week old puppies and the result were similar to those obtained with the kittens (Olson *et al.*, 1997). In all these vaccination attempts, protection of the animals against infection was not attained. No studies have been done on the induction of active immunity against *G. lamblia* specific cyst antigens.

Interestingly, *CWP2* has been shown to react with mAb 8C5 (unpublished data), which would make it a potential candidate for the development of a vaccine. However, nothing is known about the immunogenicity of *CWP2*. In this work, we show that a *CWP2*-specific, local and systemic, immune response is stimulated when mice were immunized orally with *CWP2* and CT as an adjuvant. The *in vitro* cytokine production by Peyer's patches (PP), mesenteric lymph nodes (MLN), and spleen cells will also be discussed.

MATERIAL AND METHODS

Animals

Eight week old female BALB/c and CD-1 mice were purchased from Charles River Breeding labs (St. Constant, Quebec). The BALB/c mice were used for the immunization experiments and the CD-1 mice were used for maintenance of the *G. muris* parasite.

Parasites

(i) *G. lamblia*

Trophozoites, WB strain (American Type Culture Collection no. 30957), originally isolated from a patient with chronic symptomatic giardiasis (Smith *et al.*, 1982), were used to prepare soluble antigenic extracts. Trophozoites were grown axenically in filter-sterilized TYI-S-33 (trypticase, yeast extract, iron, serum) medium adapted for *G. lamblia* (Gillin and Diamond, 1979), supplemented with 10% adult bovine serum (Sigma Chemical CO., St. Louis, USA), and 100 U/ml of penicillin (Sigma) and 100 μg/ml of dihydrostreptomycin sulphate salt (Sigma), in the absence of bovine bile at pH 7.0. Trophozoites were passaged twice weekly.

(ii) *G. muris*

Cysts were obtained from Dr. M. Belosevic (University of Alberta, Edmonton, Canada). They were stored at 4 ^{0}C in phosphate-buffered saline (PBS; pH 7.2) until mice were infected orally with 10^{3} cysts suspended in 0.5 ml of PBS.

***In vitro* encystation**

To induce *in vitro* encystation, the method of Campbell and Faubert (1994) was followed. Briefly, spent medium from fully grown *G. lamblia* cultures (72 hours) was removed and the adherent trophozoite monolayer refed with complete encystation medium (TYI-S-33, pH 7.8, supplemented with 0.25 mg/ml porcine bile (Sigma) and 0.55 mg/ml lactic acid (Sigma). *G. lamblia* trophozoites cultures were left in encystation medium for 72 hours, and after, were treated with water according to the method of Faubert *et al.* (1991) to lyse non-encysted trophozoites and non-water resistant cysts. The remaining water-resistant cysts were then collected by centrifugation at 150g for 5 minutes (min) at room temperature (RT).

Preparation of *G. lamblia* encysting cells antigenic extract

G. lamblia grown in encysting medium were used to prepare the cyst antigenic soluble extract. *G. lamblia* encysting cells were sedimented by centrifugation at 800g for 10 min at 4 ^{0}C. Encysted cells were then lysed using a Vibra-Cell sonicator (30 s bursts)(Fisher, Montreal, Canada) for 10 min in an ice bath. The sonicated cell debris was removed by centrifuging the suspension at 23 300 g for 20 min at 4 ^{0}C. The supernatant was collected and used as antigenic extract. Protein concentration was determined by the Lowry method (Lowry *et al.*, 1951).

CWP2

(i) Expression and solubility of *CWP2*

CWP2 was kindly provided by Dr. Nash from the National Institutes of Health (Laboratory of parasitic diseases, Bethesda, MD) in the form of an *Escherichia coli* clone containing plasmid

pMM109. *CWP2* protein was produced as a recombinant protein in hexa-histidine affinity tag gene fusion system. *E. coli* cells expressing *CWP2* was grown overnight at 37 °C in Luria-Bertani (LB) medium containing ampicillin. Expression was induced in a six hour (37 °C) shaking culture with 1mM isopropyl-β-D-thiogalacto-pyranoside (IPTG) for four hours. A protocol to separate the insoluble and soluble fraction of the culture was followed to determine the solubility of *CWP2*. Induced *E. coli* cells were collected by centrifuging the culture at 325g for 10 min and lysed by sonication as mentioned above. The lysate was centrifuged at 11 000g for 15 min at 4 °C. The pellet and the supernatant were both resuspended in reducing sample buffer (62.5 mM, pH 6.8, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol and 0.1% bromophenol blue).

(ii) Isolation of *CWP2* from inclusion bodies

Inclusion bodies are aggregated, dense structures of proteins produced mostly in the cytoplasm of *E. coli*, which is the result of a high expression rate of protein in an over-expression system. To determine whether *CWP2* was present in inclusion bodies, the bacteria were lysed with lysosyme and centrifuged at 12 000g for 10 min at 4 °C. The protein was found to be expressed in inclusion bodies of *E. coli*. A protocol adapted from the method of Kleid *et al.* (1981) and Marston (1987) for expression and isolation of proteins from inclusion bodies was followed. Briefly, *E.coli* expressing *CWP2* was grown overnight at 37 °C in LB containing ampicillin and induced with IPTG for four hours. The bacteria culture was centrifuged and resuspended in 100 mM NaCl, 1 mM EDTA and 50 mM Tris (pH 8) supplemented with lysosyme (Sigma, USA). After centrifugation, the pellet was frozen and thawed three times to disrupt the cells. The pellet was resuspended in ice cold 100 mM NaCl, 1 mM EDTA, 0.1% sodium deoxycholate and 50 mM Tris (pH 8). $MgCl_2$ and Dnase I (Sigma) were added with occasional mixing. Inclusion bodies were removed from

suspension by centrifugation at 10 000g for 10 min. The pellet was washed and resuspended in 1% NP-40, 100 mM NaCl, 1 mM EDTA and 50 mM Tris (pH 8). The pellet was washed a second time without 1% NP-40. The protein concentration was determined by the Lowry method (Lowry *et al.*, 1954) and the *CWP2* extract stored at -20 ^{0}C until used.

Western and Dot blotting

The analyses were carried out by using standard techniques. The *CWP2* proteins were boiled in an equal volume of reducing sample buffer and applied in volumes of 15 µl at 20 µg per lane. The technique of Laemmli (1970) was used for SDS-PAGE with the following modifications. Proteins were separated on a 4% stacking gel and 12% separating gel in a Bio-Rad protean II electrophoresis unit.

(i) Western blotting

The method of Towbin *et al.* (1979) was used. The proteins were electrophoretically transferred from gels to nitrocellulose (NC) in a Rad blotting chamber at 1 ampere for 2 hours at 4 ^{0}C. The protein transfer was checked by staining with Coomassie blue. NC strip was blocked in 5% milk for one hour at RT and then washed with PBS (Johnson *et al.*, 1984). The strips were incubated with the first antibody, mAb 8C5.C11 diluted 1:1000, for one hour at RT. It was washed again with anti-mouse horseradish peroxidase whole antibody (Amersham Pharmacia biotech, UK) diluted 1:3000. Detection was done with enhanced chemi-luminescent (ECL) western blotting detection reagents (Amersham Pharmacia biotech, UK).

(ii) Dot blotting

Dot blotting was used to determine if the *CWP2* protein expressed in *E. coli* was in the

soluble or insoluble fraction. It was used because of its high sensitivity and its rapidity of execution. The technique adapted from Yen and Webster (1982) was followed. The protein solution was added directly to the NC sheet and allowed to bind to the paper for 15 min. The antibody capture on NC was done as described in the western blotting technique.

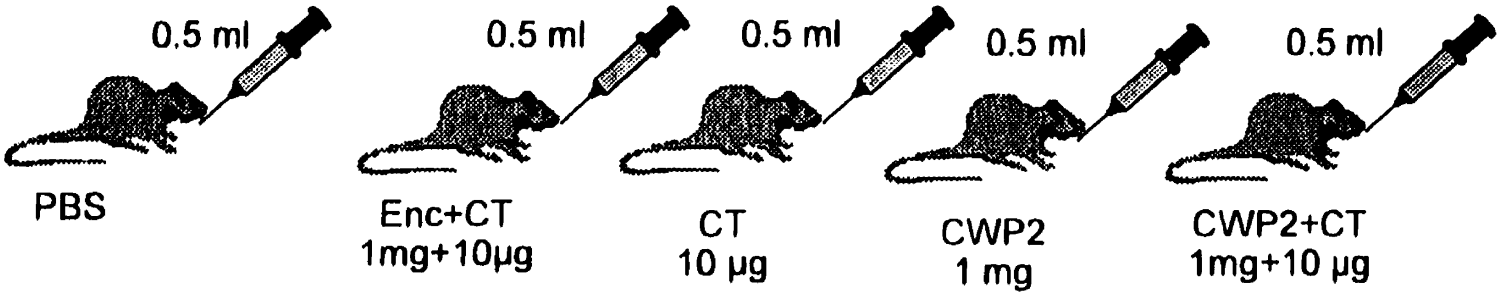
Immunizations

Five groups of BALB/c mice were immunized by oral gavage (Fig. 1a). Three groups served as control and two groups were used as test samples. The first group (four mice) was injected with PBS (pH 7.2) supplemented with 3% $NaHCO_3$ and served as the negative control: the second group (eight mice) was immunized with soluble extracts of *G. lamblia* encysting cells (Enc) mixed with the adjuvant CT (Gibco BRL, USA) and served as a positive control while the third group (four mice) was immunized with CT alone and served as a control for the adjuvant: the fourth group (eight mice) was immunized with *CWP2* alone: and the fifth group (eight mice) was immunized with *CWP2* plus CT. The total volume of inoculation given to each mouse was 0.5 ml. Therefore, the total protein concentration of *CWP2* or *G. lamblia* encysting cells given to each mouse was 1 mg per inoculation. The concentration of CT given to the mice was 10 µg per inoculation. Mice were immunized every week for a period of four weeks and were sacrificed one week after the last immunization (Fig. 1b). This experiment was repeated twice with a different population of mice.

Isolation of *G. muris* cysts from fecal pellets

Six CD-1 mice were infected orally with 10^3 *G. muris* cysts in 0.5 ml of PBS solution. For collection of feces, individual mice were placed in separate cages and the fecal pellets excreted over

A) Immunization:



B) Schedule:

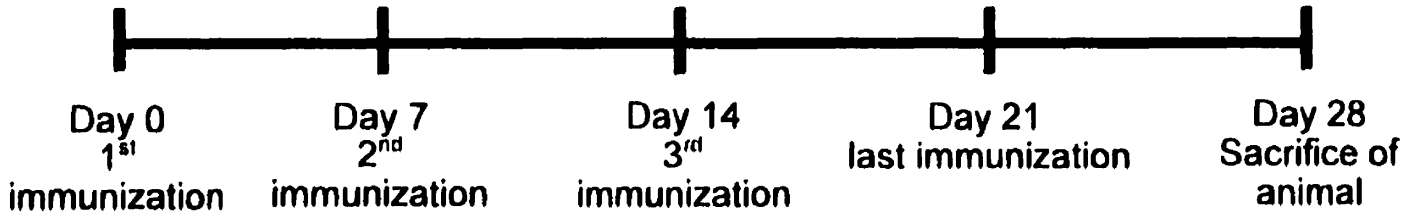


FIG. 1. Immunization protocol. (A) Five groups of mice were immunized orally with an inoculation of 0.5 ml. Three of these groups serve as control: negative control, injected with PBS; positive control, injected with a soluble extract of Enc + CT; and adjuvant control, injected with CT adjuvant. The remaining two were immunized with *CWP2* and *CWP2* + CT. The protein concentration of Enc and *CWP2* was 1 mg per inoculation while the concentration of CT was 10 µg per inoculation. (B) Mice were immunized orally with 4 doses at one week interval. The mice were sacrificed one week following the last immunization.

a one hour period were collected in 12 x 75 mm glass tubes. Cysts were isolated by a sucrose gradient centrifugation technique adapted by Belosevic and Faubert (1986). Briefly, feces were collected, emulsified in PBS, layered on sucrose (specific gravity 1.12), and centrifuged at 400g for 15 min. Cysts migrating at the PBS-sucrose interface were removed, washed in PBS, sedimented by centrifugation at 600g for 10 min, and resuspended in 1 ml of PBS. Cysts were then counted with a Spencer Bright Line hemacytometer.

Sample collection

Blood and fecal pellets were collected to determine the presence of systemic and local antibody responses.

(i) Blood

Immunized BALB/c mice were anaesthetized and bled from the orbital plexus at the time of sacrifice. Blood samples of approximately one ml were collected for each mouse. Blood was kept at 4 ^{0}C over night to separate the serum and the erythrocytes. The serum was collected by centrifuging the blood samples at 800g for 10 min and stored at -70 ^{0}C.

(ii) Fecal pellets

Before sacrificing the mice, fresh fecal pellets were collected from each immunized mouse. In infected mice, feces were collected at 14 days post-infection (acute phase), 29 days post-infection (early elimination phase) and 35 days post-infection (late elimination phase). The pellets were weighed (approximately 0.15 g) and frozen at -20 ^{0}C. Soluble fecal extracts were prepared following the technique of Steidler *et al.* (1998) to measure the presence of antibodies in feces. The soluble extracts were prepared in a microcentrifuge tube by adding 1 ml of PBS containing 1% bovine serum

albumin (BSA) (Boehringer Mannheim, Germany) and 1 mM of freshly added phenylmethyl sulfonylfluoride (PMSF) (Gibco BRL, USA) per 0.1mg of fecal material. The tubes were incubated overnight at 4°C to softened up the feces. Feces were then vigorously mixed by vortex to disrupt and suspend all solid matter. The samples were centrifuged at full speed in a microcentrifuge for 5 min. The supernatants were collected and stored at -20 °C.

ELISA assay

ELISA assays were carried out by using a standard technique as described by Miles and Hales (1968). Briefly, wells were coated with 50 µl of cyst antigen *CWP2* in PBS and the ELISA plates (Falcon, VWR) were incubated at 4 °C overnight and washed with PBS-Tween. Plates were blocked with PBS-1%BSA (200 µl/well). Both the sera samples and the soluble fecal extracts samples were diluted in PBS-Tween and tested in triplicate. Each plate was controlled by using samples from non-immunized mice of the same age and batch as experimental mice. After incubation and washing, the second antibodies, horseradish peroxidase conjugated anti-mouse IgA (0.4 mg/ml-1:2000), IgM (0.5 mg/ml-1:30 000), IgG1 (0.7 mg/ml-1:3000) and IgG2a (0.7 mg/ml-1:3000) were added at optimized dilutions. After a one hour incubation at RT, plates were washed. 2.2- Azino-di-(3-ethyl Benzthiazoline)-sulfonate Diammonium salt (ABTS) (ICN Biochemicals, USA) and hydrogen peroxide were added as the developing substrate. The plates were read at a wavelength of 405 nm with a microplate reader (Mandel Scientific, Guelph, Canada).

Preparation of spleen, Peyer's patches and mesenteric lymph nodes cells

After mice were sacrificed, spleens were removed aseptically and pooled. The preparation

of spleen cells for culture followed the method of Djamiatun and Faubert (1998). Briefly, the cells were forced through a stainless steel mesh placed in a petri dish containing 10 ml RPMI 1640 (Gibco, Grand Island, New York, USA), 1% fetal bovine serum (FBS)(Gibco BRL, USA), 100 U/ml penicillin and 100 µg/ml dihydrostreptomycin sulphate salt (Sigma) and 2g/l $NaHCO_3$. The cell suspension was washed three times with RPMI 1640 at 1500 RPM for 10 min at 4 ^{0}C. Erythrocytes were lysed with red blood cells (RBC) lysing buffer. The membrane debris was removed by filtering the cell suspension through sterile gauze. The cell viability was determined by the trypan blue (Sigma) dye exclusion test. Only suspensions containing >90% viable cells were used. The concentration of the cells was adjusted to 5 x 10^6 in RPMI 1640 supplemented with 10% fetal bovine serum.

MLN and Peyer's PP cells were removed aseptically and pooled together. The cells were prepared in a similar manner as spleen cells except that 400 U/ml penicillin and 400 µg/ml dihydrostreptomycin sulphate salt (Sigma) were added to the RPMI 1640. Cells were not forced through a wire mesh but crushed with a pestle instead. Only suspensions containing at least 90% viable cells were used.

Cell culture for cytokine release

Cells of PP, MLN and spleens were cultivated for cytokine release. One-milliliter volumes of spleen, MLN and PP cells (5 x 10^6/ml) in supplemented RPMI 1640 were each cultured in 24-well plates (Falcon, VWR). The method of Dillon *et al.* (1986) was followed for the stimulation of cells for cytokine release. Three wells were used for each suspension: one was used as a control, one was stimulated with 5 µg of Concanavalin (Con) A (ICN Biochemicals, USA), and one was stimulated

with 12 μg of *CWP2* antigen. The cell cultures were incubated for 48 h at 37 ^{0}C in 5 % CO_2 atmosphere. The supernatants from triplicate wells were collected and stored at -70 ^{0}C until used for the cytokine assay.

Cytokine ELISA assay

The method followed by Djamiatun and Faubert (1998) was used for the cytokine assays with the following modifications. The two-site sandwich ELISA was used to measure the cytokines: Interferon (IFN)-γ, representing Th1 cytokines, and IL-4 and IL-5, representing Th2 cytokines. Monoclonal antibodies against these cytokines were purchased from Pharmingen (San Diego, USA). Wells of a 96-well microtiter plate (Immulon-2 "U" bottom: VWR, Montreal, Canada) were coated with first anti-cytokine mAb overnight at 4 ^{0}C. Serum samples and fecal extract samples tested were used undiluted. Biotinylated second anti-cytokine mAb were added to each well and incubated at RT for one hour. After incubation of Avidin Peroxidase (1:500) (Sigma) for 30 min, plates were washed and citric buffer plus OPD (o-Phenylenediamine) (Sigma) and hydrogen peroxide were added as developing substrate. Sulfuric acid was added to stop the developing reaction and the plates were read in a microplate reader at wavelength of 405 nm. The concentration of each cytokine was calculated from standard curves prepared using known concentrations of recombinant murine IFN-γ (rIFN-γ), rIL-4 or rIL-5 (Pharmingen).

Statistical analysis

Statistical significance was determined using a t-test. The probability level of $P<0.05$ was considered significant.

RESULTS

Expression and solubility of *CWP2*

CWP2 was successfully expressed and produced as a recombinant protein with an hexa-histidine affinity tag, using a PQE-30 gene fusion vector. A Dot blotting assay was done with the cyst specific mAb 8C5.C11 to determine if *CWP2* expressed in *E. coli* was found in the soluble or insoluble fraction (Fig. 2). A lysate of *E. coli* cells expressing *CWP2* acted as a positive control and reacted positively with the mAb (Fig. 2 panel A). The vector PQE-30 expressing the plasmid without the gene encoding for *CWP2* acted as a negative control and did not react with the mAb (Fig. 2 panel B). A soluble and an insoluble fraction of *E.coli* cells expressing *CWP2* were tested against mAb 8C5.C11. No reaction was observed between the soluble fraction and the mAb (Fig. 2 panel C) but a positive reaction was observed between the insoluble fraction and the mAb (Fig. 2 panel D). This demonstrates that recombinant (r)-*CWP2* was found to be an insoluble protein.

Isolation of *CWP2* from inclusion bodies

CWP2 was isolated from inclusion bodies using a standard technique to isolate protein antigens from bacterial over-expression system (Fig 3). In a Western blotting assay, the isolated sample of *CWP2* from inclusion bodies reacted intensely with the mAb 8C5.C11 (Fig. 3 lane B). A plasmid not encoding for any protein was also tested with the mAb as a negative control, as was a plasmid encoding for an *Entamoeba histolytica* cyst protein; both of them did not react positively (Fig. 3 lane A and C, respectivelly). A positive control was also used which was a cell lysate of *E. coli* expressing the r-*CWP2* and was found to react positively with the mAb (Fig. 3 lane D).

However, despite several attempts, it was not possible to obtain one single band of 39 KD, which corresponds to the molecular weight of *CWP2*. One of the reason why a clear band of *CWP2* could not be observed could be because *CWP2* tends to often aggregate with another molecule of *CWP2*. Hence, to assure that the positive reaction observed between *CWP2* and the mAb 8C5.C11 was not due to non-specific binding of the mAb, a Western blotting assay was done with the mAb PB-1801 and PB-1802 (Fig. 4). These mAb are specific to protein p53 (cellular tumour-suppressor protein of 53 KD) and are of the same isotype as mAb 8C5.C11, which is IgG1. Monoclonal antibodies PB-1801 and PB-1802 reacted with 53 KD bands representing the positive control p53 proline and p53 arginine (Fig. 4 lane B and C, respectivelly). No reaction was observed between *CWP2* isolated from inclusion bodies and Pb-1801 and 1802 (Fig. 4 lane A). This indicated that the positive reaction observed between *CWP2* and mAb 8C5.C11 is not due to antibody cross-reactivity.

CWP2-specific local antibodies

BALB/c mice were immunized orally with *CWP2*, *CWP2* + CT, Enc + CT or CT alone. PBS was also administered to a group of mice which acted as the negative control group. Mice were immunized once a week for a period of 4 weeks. One week after the last immunization, we collected fecal pellets for analysis of the specific antibody responses. The results are expressed as mean of O.D. levels ± standard error of the mean (SEM). IgA was the only isotype detected in fecal extracts by ELISA (Fig. 5a,b). The group of mice immunized with *CWP2*, *CWP2* + CT or the positive control, Enc + CT, were all significantly positive ($P<0.05$) for the presence of *CWP2*-specific fecal IgA antibodies compared to the group administered PBS, the negative control group (Fig. 5a,b). The negative control group is represented by the horizontal line observed in the graphs. No significant

differences were observed in the O.D levels of IgA between the group of mice immunized with *CWP2*, *CWP2*+CT or Enc + CT. The group of mice immunized with CT alone did not show presence of *CWP2*-specific fecal IgA (Fig. 5a,b). *CWP2*-specific IgM, IgG1, and IgG2a were not detected in fecal extracts of any groups of immunized mice (data not shown).

CWP2-specific systemic antibodies

Serum samples were collected from the same group of immunized BALB/c mice as mentioned above and on the same day as fecal pellets were collected. Serum samples of mice immunized with *CWP2* + CT showed the presence of antibodies specific to the *CWP2* antigen, as determined by ELISA. The results are expressed as mean of O.D. levels ± SEM. The group *CWP2* + CT was significantly positive ($P<0.05$) for the presence of *CWP2*-specific IgA, IgG1 and IgG2a antibodies but negative for the presence of IgM when compared to the negative PBS control group (represented by the horizontal line in the graph) (Fig. 6a,b,c,d). No antibodies were detected in the serum of mice immunized with CT alone or Enc + CT (Fig. 6a,b,c,d). The group of mice immunized with *CWP2* alone was found negative for the presence of antibodies in the first trial but was found to be significantly positive ($P<0.05$) for the production of *CWP2*-specific IgA and IgG2a antibodies in the second trial (Fig. 6a,d).

Fecal antibody responses in _G. muris_ infected mice

Fecal extracts of *G. muris* infected mice were found to be significantly positive for the production of *CWP2*–specific IgA (Fig. 7). The fecal extracts were also found to be significantly positive ($P<0.05$) for the production of encysting cells-specific IgA (data not shown). Specific IgA

antibodies to *CWP2* were detected in feces of infected mice collected at 14, 28 and 35 days post-infection (Fig. 7). *G. muris* infected mice are able to recognize and raise a specific IgA antibody response against *G. lamblia CWP2* (Fig. 7).

Cytokine production by the PP and MLN cells obtained from immunized mice

Con A and *CWP2* induced *in vitro* cytokine production by PP and MLN is shown in Fig. 8. When Con A (5μg/ml) was used to stimulate PP and MLN cells removed from immunized mice, IFN-γ and IL-4 were detected in the culture supernatant (Fig. 8a,b). Levels of neither IFN-γ nor IL-4 were significantly higher ($P<0.05$) in immunized mice, except mice administered CT, when compared to non-immunized mice.

However, PP and MLN cells challenged *in vitro* with *CWP2* (12 μg/ml) released very low levels of both IFN-γ(ca. 0.1 ng/ml) and IL-4 (ca. 0.02 ng/ml)(Fig. 7a,b). No immunized groups were found significantly positive ($P<0.05$) for the production of cytokines when compared to non-immunized mice (Fig. 8a,b).

Cytokine production by spleen cells obtained from immunized mice

Con A and *CWP2* induced *in vitro* cytokine production by spleen cells is shown in Fig. 9. When Con A (5μg/ml) was used to challenge spleen cells from immunized mice, IFN-γ, IL-4 and IL-5 were detected in the culture supernatant (Fig. 9a,b,c). As in PP and MLN cells, levels of neither IFN-γ, IL-4 nor IL-5 were significantly higher ($P<0.05$) when compared to non-immunized mice. Only mice administered CT was significantly positive ($P<0.05$) for the production of IL-5. Levels of IFN-γ (ca. 2.2 ng/ml) were more than 4-fold higher than levels of IL-4 (ca. 0.5 ng/ml) and IL-5

(ca. 0.1 ng/ml).

However, as in PP and MLN cells, spleen cells challenged in vitro with CWP2 (12 μg/ml) released very low levels of IFN-γ (ca. 0.5 ng/ml), IL-4 (ca. 0.2 ng/ml) and IL-5 (ca. 0.02 ng/ml) when compared with levels of cytokines released when challenged with Con A (Fig. 9a,b,c). None of the immunized groups of mice were found to be significantly positive ($P<0.05$) for the production of any cytokines when compared to non-immunized groups of mice.

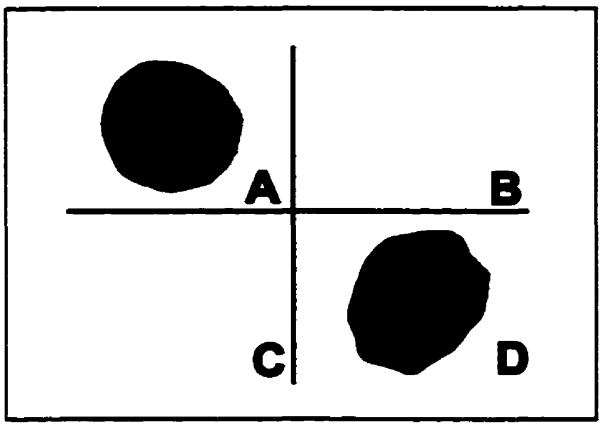


FIG. 2. Dot-blotting assay of *CWP2* reacting with mAb 8C5. In order to determine the solubility of *CWP2*, a protocol to separate the soluble and insoluble fraction of the culture was followed. Panel A represents the positive control which is a cell lysate of *E. coli* expressing r-*CWP2*. Panel B represents the negative control which is the vector PQE-30 expressing the plasmid without the gene encoding for *CWP2*. Panel C represents the soluble fraction of *E.coli* cells expressing r-*CWP2* and panel D is the insoluble fraction *E.coli* cells expressing r-*CWP2*.

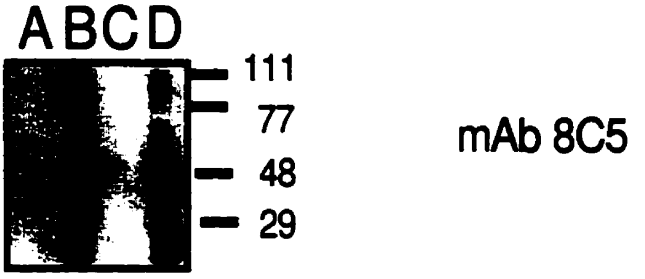


FIG. 3. Western blotting assay of recombinant *CWP2* reacting with mAb 8C5. In lane A, *E. histolytica* proteins isolated from inclusion bodies act as a negative control. Lane C also represents a negative control which is the vector PQE-30 expressing the plasmid without the gene encoding for *CWP2*. Lane D is a positive control which is a cell lysate of *E. coli* expressing recombinant *CWP2*. Lane B is recombinant *CWP2* isolated from inclusion bodies. Standard molecular weights are shown on the right (KD).

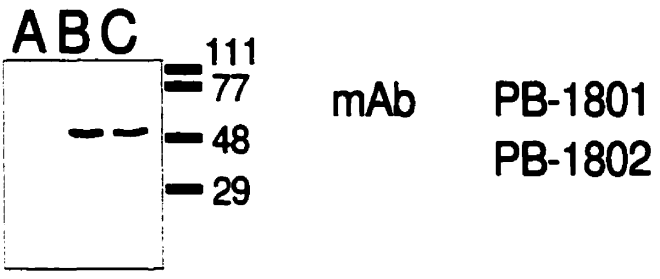


FIG. 4. Western blotting assay of p53 proteins reacting with specific antibodies. mAb PB-1801 and PB-1802 which are specific to the protein p53 were used to confirm the specificity between *CWP2* and mAb 8C5.C11. These mAb were of the same isotype as mAb 8C5.C11. In lane A, there is recombinant *CWP2* isolated from inclusion bodies. In lane B, there is a positive control which is p53 in the arginine form. In lane C, there is p53 in the proline form, another positive control. Standard molecular weights are shown on the right (KD).

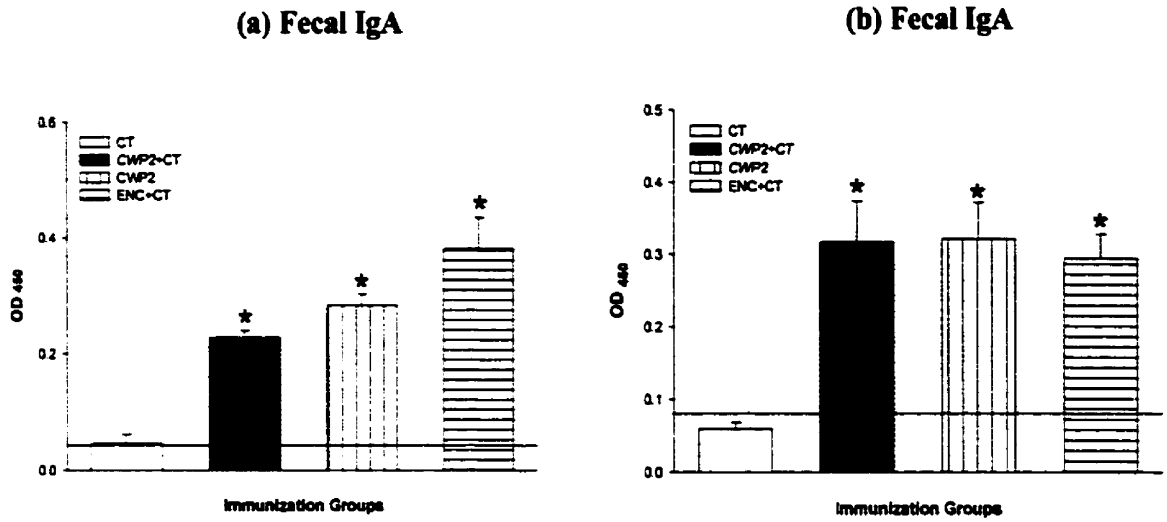
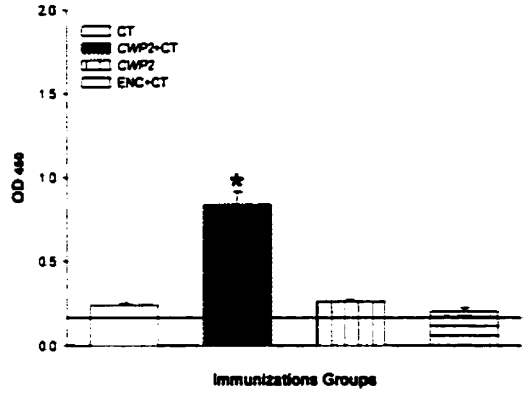
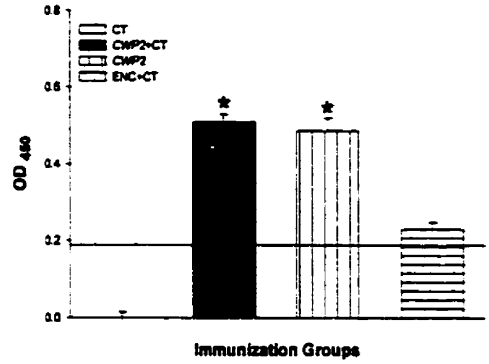


FIG. 5. *CWP2*-specific fecal antibody responses. Mice were immunized orally with either CT (adjuvant control), *CWP2* + CT, *CWP2* or encysting cells + CT. The horizontal line represents the control group which is mice injected with PBS. Mice were immunized every week for a period of four weeks and feces were collected one week after the last immunization. (a) is the first set of immunization while (b) is a replicate of the immunization experiment with a different population of mice. Results are expressed as mean of O.D. levels ± SEM. *Statistically significant at $P<0.05$.

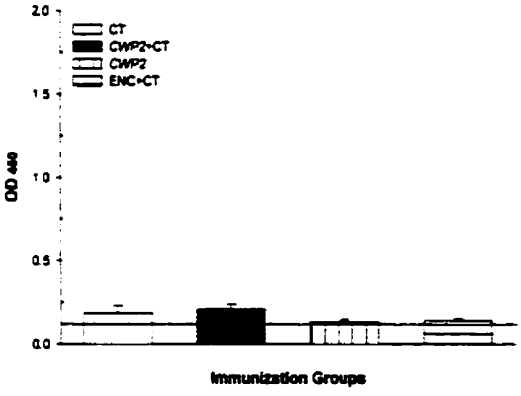
1st set of immunization experiment **2nd set of immunization experiment**

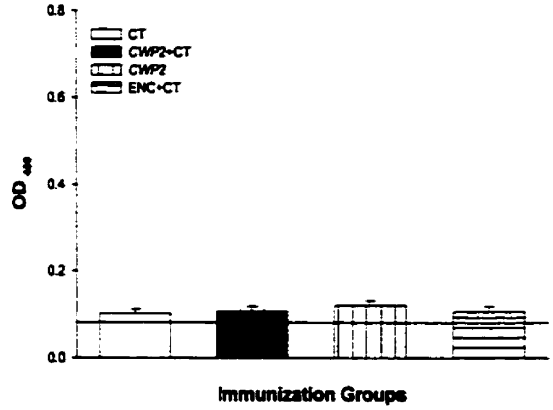
(a) Serum IgA



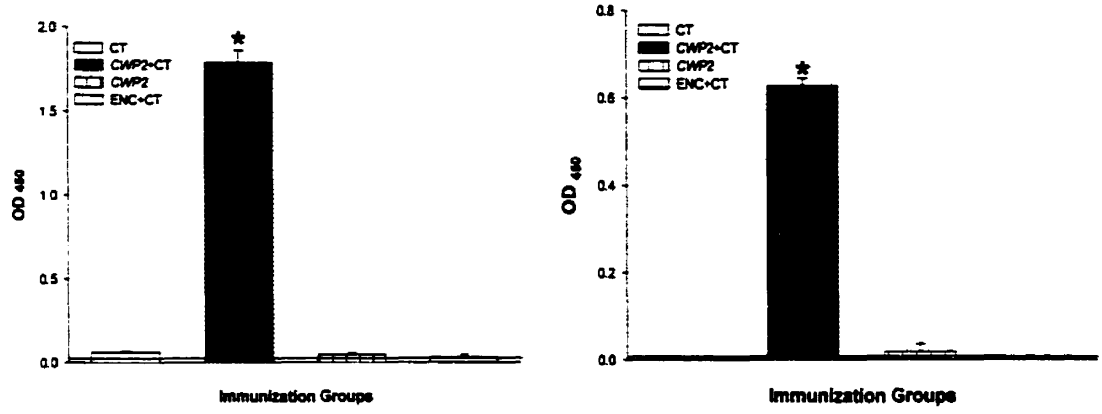


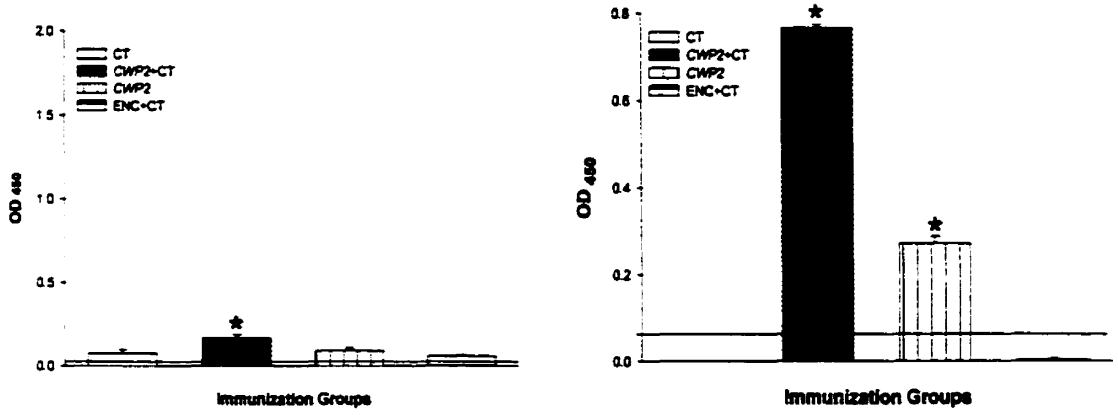
(b) Serum IgM





(c) Serum IgG1





(d) Serum IgG2a

FIG. 6. *CWP2*-specific serum antibody responses. Mice were immunized orally with either CT (adjuvant control), *CWP2* + CT, *CWP2* or encysting cells + CT. The horizontal line represents the control group which are mice injected with PBS. Mice were immunized every week for a period of four weeks and serum was collected one week after the last immunization. Results are expressed as mean of O.D. levels ± SEM. *Statistically significant at $P<0.05$.

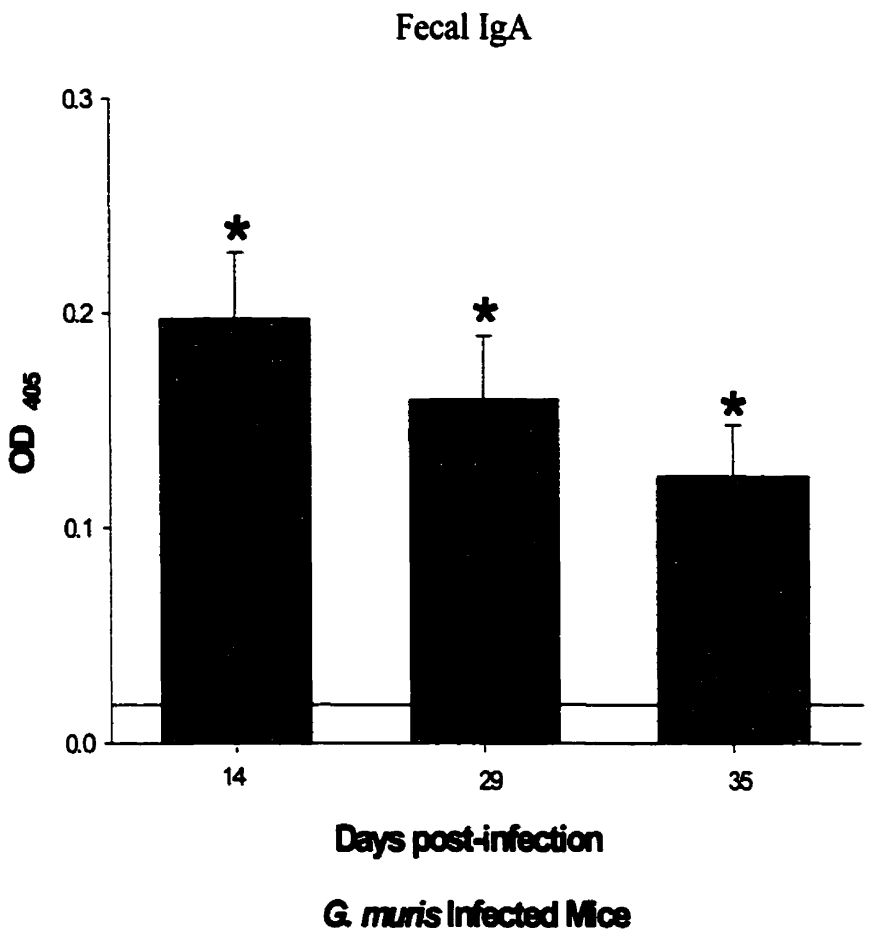


FIG. 7. *G. lamblia CWP2*-specific fecal IgA Ab responses in mice infected with 10^3 *G. muris* cysts. Fecal pellets were collected at 14 days post-infection (acute phase), 29 days post-infection (early elimination phase) and 35 days post-infection (late elimination phase). The horizontal line represents the control group which were non-infected mice. Results are expressed as mean of O.D. levels ± SEM. *Statistically significant at $P<0.05$.

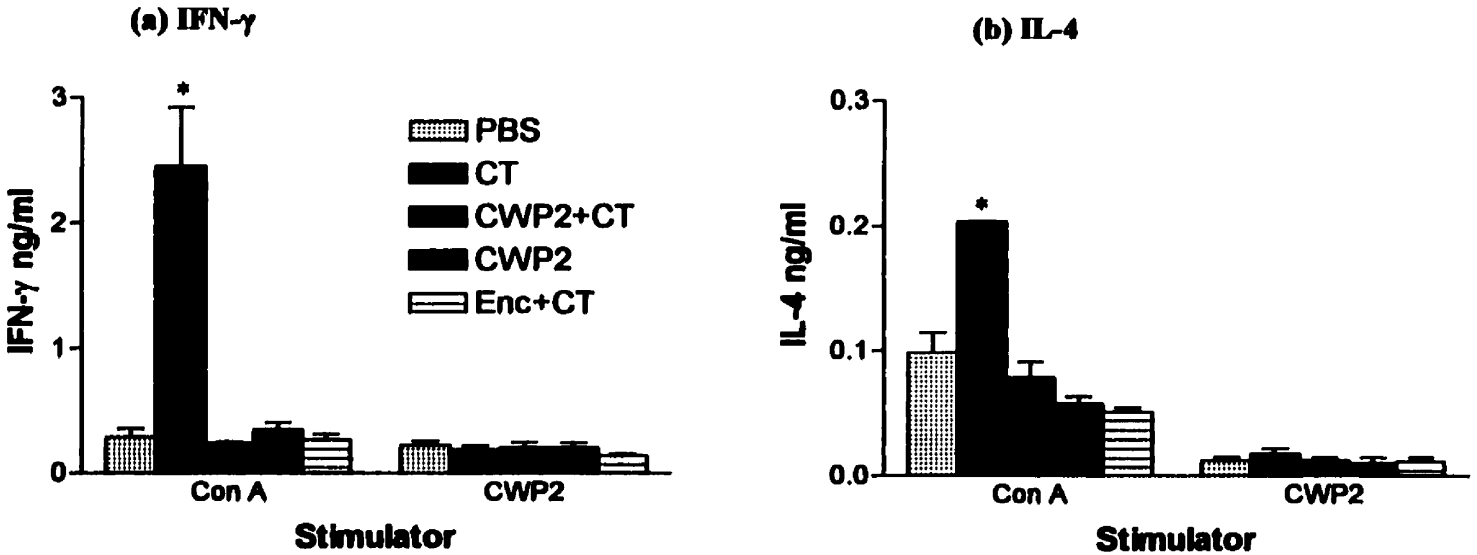


FIG. 9. *In vitro* cytokine release by PP and MLN cells from immunized mice in response to Con A (5 μg) or *CWP2* (12 μg). Mice were immunized orally with either PBS (negative control), encysting cells + CT (positive control), CT (adjuvant control), *CWP2* or *CWP2* + CT. Mice were immunized every week for a period of four weeks and spleen cells were harvested one week after the last immunization. Tissue culture supernatants were assayed for IFN-γ (a), IL-4 (b) after 48 h. The lower limits of sensitivity in the assays were 0.21 ng/ml for IFN-γ and 0.012 ng/ml for IL-4. Results are expressed as mean ± SEM. *Statistically significant at $P<0.05$.

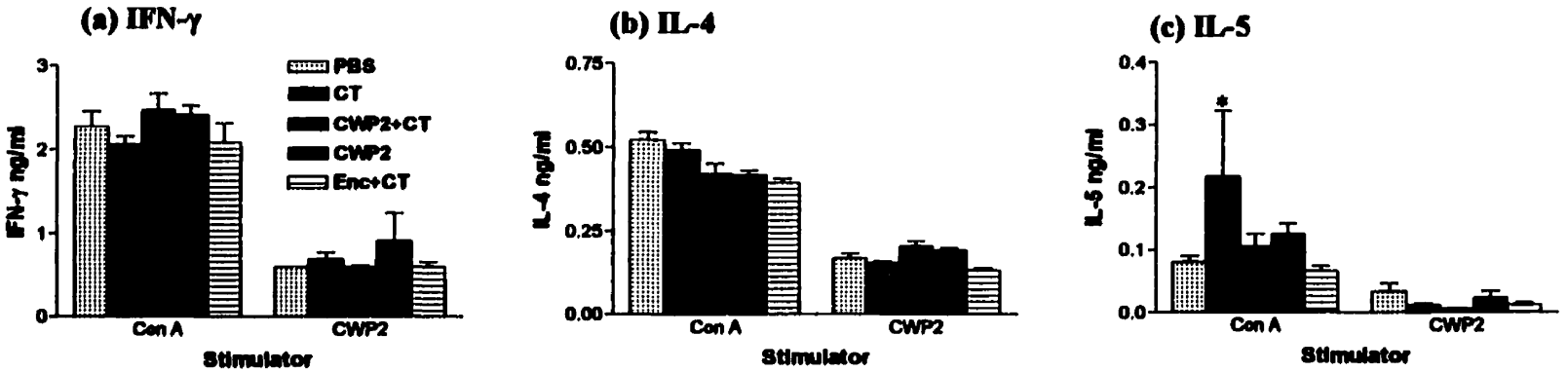


FIG. 8. *In vitro* cytokine release by spleen cells from immunized mice in response to Con A (5 μg) or *CWP2* (12 μg). Mice were immunized orally with either PBS (negative control), encysting cells + CT (positive control), CT (adjuvant control), *CWP2* or *CWP2* + CT. Mice were immunized every week for a period of four weeks and spleen cells were harvested one week after the last immunization. Tissue culture supernatants were assayed for IFN-γ (a), IL-4 (b) and IL-5 (c) after 48 h. The lower limits of sensitivity in the assays were 0.25 ng/ml for IFN-γ, 0.11ng/ml for IL-4 and 0.01 ng/ml for IL-5. Results are expressed as mean ± SEM. *Statistically significant at $P<0.05$.

DISCUSSION

The immunogenicity of BALB/c mice with a sonicated extract of *in vitro*-generated *G. lamblia* cysts produced a mAb (8C5.C11) which recognized proteinaceous cyst antigens (Campbell and Faubert, 1994). When added to *G. lamblia* encysting cultures, before the appearance of cysts, mAb 8C5.C11 caused a significant reduction in the number of water-resistant cysts produced *in vitro*. The first objective of this study was to determine if *CWP2*, a gene product which reacts with mAb 8C5.C11 in an immunoblot assay, was immunogenic when it was given to mice by oral gavage.

The gene encoding for *CWP2* was cloned by Lujan *et al.*(1995). They have studied the structural and biochemical properties of *CWP2*, but have never determined its solubility in an over-expression system. In this sudy, I have produced a synthetic *CWP2* protein as a recombinant protein linked with an hexa-histidine affinity tag. It was shown that *CWP2* was found to be insoluble when expressed in *E. coli* cells. When *CWP2* was produced in an over-expression system, it was deposited in inclusion bodies. Inclusion bodies have been found to be produced in the cytoplasm of *E. coli* cells. There are many reasons that may explain the presence of these structures: the heterologous nature of the protein; protein is expressed at a higher rate; relatively more hydrophobic proteins which aggregate inter-molecularly as a result of non-covalent association; and finally, chaperones like helper proteins are either inadequately available or absent (Mukhopadhyay, 1997). All of these above reasons could explain why *CWP2* was found in inclusion bodies. *CWP2* is heterologous, it is expressed in an overexpression system, and it was shown to demonstrate a high percentage of hydrophobicity. Also, BiP (binding protein), an endoplasmic reticulum molecular chaperone has been found to maintain *CWP2* in a soluble form inhibiting an early and unproductive polymerization

(Lujan *et al.*, 1997). However, BiP might not be made available or present in *E. coli* cells.

In spite of several attempts and modifications, the Western blot assay performed with *CWP2* isolated from inclusion bodies never showed a clear 39 KD band under denaturing condition. This could have been due to the degradation of the protein. However, this possibility was eliminated since a cocktail of proteinase inhibitors was used. It has been reported that *CWP2* could aggregate with another molecule of *CWP2* (Lujan *et al.*, 1997), which could explain the fused band observed on the film. For this reason, necessary negative controls, such as the vector not expressing *CWP2* or expressing an *E. histolytica* protein, were used to assure that *CWP2* was successfully isolated from inclusion bodies (Fig. 2 and 3).

It has been shown that humoral antibodies play an important role in the elimination of trophozoites from the intestine (Brown *et al.*, 1972; Snider *et al.*, 1985). Our study showed the presence of *CWP2*-specific IgA antibodies in fecal pellets of immunized mice. Namely, both group of mice immunized with *CWP2* or *CWP2* + CT induced a *CWP2*-specific IgA response in the feces. These results revealed that a specific local antibody response was raised against *CWP2*. S-IgA has been found to be the key isotype in the antibody-mediated clearance of the *Giardia* trophozoites (Snider *et al.*, 1988, Faubert, 1996, 2000). Therefore, it can be expected that S-IgA would have a similar function in the reduction of cyst formation. In this present study, IgM, IgG1 and IgG2a were not found in fecal pellets. This is supported by the fact that proteolytic cleavage of endoluminal IgG and S-IgM by endogeneous digestive enzymes occurs, leading to Fab fragments (Bouvet and Fischetti, 1999). In contrast, the structure of S-IgA affords it resistance to most proteases.

Specific antibodies to *CWP2* of the IgA, IgG1 and IgG2a isotypes were detected in the serum of immunized mice. This observation confirms that the oral immunization not only stimulated the

local immune response but the systemic immune response as well. These results are interesting because it has been shown that serum IgA, IgM and IgG specific antibodies are involved in the clearance of trophozoites in the intestine (Susano *et al.*, 1993; Kumkum *et al.*, 1988; Snider *et al.*, 1985). The presence of serum IgG stimulates our interest because it has been reported that IgG isotype antibodies are highly produced by memory B cells (Bachmann *et al.*, 1994). For example, levels of IgG antibodies remain elevated for months or even years after primary *Giardia* infection (Gilman *et al.*, 1985). However, no correlation between serum IgG levels and protection has been demonstrated in giardiasis. In the present study, IgM antibodies were not detected in the serum of immunized mice. This could be explained by the fact that it is the first immunoglobulin class produced in a primary response to an antigen. Considering that the ELISA assays were performed one month after the first immunization, it was not expected to detect any IgM antibodies. It has also been observed in human giardiasis that IgM antibody titers increase in early infection but return to normal levels three weeks after infection (Goka *et al.*, 1986; Nash *et al.*, 1987). Between the first and second set of immunization experiments, differences were observed in the detection of antibodies in the group of mice immunized with only *CWP2*. In the first set of immunization experiments, no antibodies were detected whereas in the second set of immunization experiment *CWP2*-specific IgA and IgG1 were detected in the serum. Speculation could be made that even though inoculation of 1 mg of proteins was given to each mouse, the concentration of *CWP2* antigen received by each mouse may have differed which would have influenced the immune response. This would be due to the fact that *CWP2* was not injected to mice in a purified form.

It was shown that local *CWP2*-specific antibody immune response could be induced when immunizing mice with *CWP2* alone. However, inconsistency in the stimulation of a systemic

specific antibody immune response was observed. On the other hand, *CWP2* + CT seemed to give more consistent results, which means that at all times it stimulated both a *CWP2*-specific local and sytemic immune response. No significant differences were observed in the fecal IgA response between the mice immunized with *CWP2* or *CWP2* + CT. This result indicates that CT did not show significant adjuvancy to *CWP2* in the feces, although CT is a potent mucosal adjuvant which stimulates IgA response to unrelated protein antigens when co-administered orally (Elson and Ealding, 1984). On the other hand, systemically, CT did show significant adjuvancy to *CWP2* because mice immunized with *CWP2* + CT showed increased antibody immune responses significantly higher than mice immunized with *CWP2* alone.

Because *CWP2* was not used in a purified form, questions arose as to whether or not the observed antibody responses were specific to *CWP2* or *E. coli* cell debris. To eliminate the speculations that the response was specific to *E. coli*, we did ELISA assays specific to encysting cells of *G. lamblia* (which are free of *E. coli* debris). The presence of antibodies was found to be significantly positive in immunized mice and the results were similar to those obtained in ELISA assays specific to *CWP2* antigen. This implies that the antibody response was not specific to *E. coli* but to *CWP2*.

Heyworth *et al.* (1987) have shown that CD4+ T helper (Th) cells are required for clearance of *Giardia* cysts from the intestine. To confirm activation of a Th1 or Th2 response, we measured the *in vitro* production of cytokines. Our studies demonstrated that, when PP and MLN cells were stimulated with Con A, the release of IFN-γ and IL-4 was detectable, but not IL-5. Considering the fact that IL-5 is a potent stimulator of the IgA production (Sonada *et al.*, 1992) and that a strong IgA response was observed in immunized mice, detectable levels of IL-5 would have been expected.

However, even though IL-5 stimulates the IgA production, it was demonstrated that the production of IL-4 was essential to induce production of specific IgA (Kopf *et al.*, 1993; Okahashi *et al.*, 1996). It could be hypothesized that IL-5 might not be totally necessary for IgA production and acts in synergy with IL-4. As for spleen cells, IFN-γ, IL-4 and IL-5 were all released *in vitro* when stimulated with Con A. The presence of S-IgA (in the feces) and serum IgA, in addition with the detection of IL-4 and IL-5, suggest an involvement of a Th2 mediated immune response because these cytokines are found to up-regulate the production of IgA (Kopf *et al.*, 1993; Okahashi *et al.*, 1996; Sonada *et al.*, 1992). However, the contrasting patterns of serum IgG isotypes production in the serum and the dichotomy of Th1 (IFN-γ) and Th2 (IL-4 and IL-5) subsets implies a differential involvement of both Th subsets locally (PP and MLN) and systemically (spleen). Briefly, IL-4 from Th2 cells and IFN-γ from Th1 cells up-regulate the production of IgG1 (Vitetta *et al.*, 1985) and IgG2a (Finkelman *et al.*, 1988), respectively.

In PP, MLN and spleen cells, IFN-γ levels were higher than levels of IL-4 and IL-5. In addition to playing a role in the control of *G. muris* infection (Venkatesan *et al.*, 1996), IFN-γ with the help of macrophages, has been shown to play a central role in the killing of the parasite *in vitro* (Belosevic and Daniels, 1992). Intestinal macrophages, activated by IFN-γ, from mice infected with *G. muris* have been shown to phagocytize trophozoites (Owen *et al.*, 1981). This response was enhanced in the presence of immune serum which suggests that an antibody-dependant cell-mediated cytotoxicity (ADCC) is involved in the phagocytosis of trophozoites by macrophages (Hill and Pohl, 1990). IFN-γ could be involved in an ADCC, possibly resulting in the killing of encysting trophozoites in the intestine. It could be speculated that the S-IgA activated by IL-4 and IL-5 would bind to the encysting trophozoites and that IFN-γ would activate macrophage activity to phagocytize

the encysting trophozoites, resulting in an ADCC response working to stop cyst formation. This type of activity has been observed in HIV infected cells where treatment of these cells with IFN-γ resulted in significant enhancement of ADCC and lysis of HIV cells (Jewett and Bonavida, 1990).

Levels of neither IFN-γ, IL-4 nor IL-5 released by PP, MLN or spleen cells, when stimulated with Con A, were significantly higher in mice immunized with *CWP2* or *CWP2* + CT as compared to non-immunized mice. These results could be explained by the fact that Con A is a mitogen known for its polyclonal activation. Hence, it could be expected that levels of cytokines released from immunized mice would not be different from the levels released by non-immunized mice. It is still unclear wether the cytokine expression patterns seen in mitogen-stimulated T cells will be the same as those observed after physiological recognition of antigens (Karulin *et al.*, 2000). Thus, the *in vitro* stimulation of T cells with Con A may reflect the Th subset population or the Th subset stimulation.

Our findings showed that spleen, PP and MLN cells from immunized mice failed to release significant levels of IL-4, IL-5 and IFN-γ when stimulated with *CWP2 in vitro*. Djamiatun and Faubert (1996) obtained similar results when they challenged T lymphocytes from *G. muris* infected or *G. lamblia* immunized mice with trophozoite antigens. These investigators were unable to detect measurable levels of IL-4, IL-5 and IFN-γ. Venkatesan *et al.* (1996) also reported that MLN cells from *G. muris* infected BALB/c mice did not produce detectable levels of both IL-5 and IFN-γ when stimulated with *G. muris* antigens. It is possible that the triggering of CD4+ T cell subset by *CWP2* was very weak. However, this is highly unlikely considering the antibody production observed in the feces and serum. It could be speculated that the reason for the low levels of cytokines observed when T lymphocytes are stimulated by *CWP2* antigen, would be that *CWP2* does not stimulate a

polyclonal activation like the mitogen Con A does. It has recently been brought up that lymphocyte polyclonal activation could be responsible for the poor success of effective parasite vaccine development (Reina-San-Martin *et al.*, 2000). Parasite antigens can obliterate specific immune responses by triggering the machinery of polyclonal lymphocyte response, thus resulting in a general lack of specificity of antibody or T cell responses to the antigens during infection and in the immuno-suppressive state that follows. For example, inhibition of humoral responses by substances derived from *Trypanosoma musculi* has been shown in mice (Albright and Albright, 1981). Also, the polyclonal activation of gamma delta T cells has been reported to have a potential role in the maintenance of the B cell hypergammaglobulinemia after *Plasmodia falciparum* infections in humans (Ho *et al.*, 1994). *CWP2*, possibly not being a polyclonal activator, would be very promising for the development of a vaccine against the cyst formation. However, in order to show that *CWP2* is not a polyclonal activator of T lymphocytes, a complete analysis of the type of immune activities such as non-specific immune responses, inflamation reaction, auto-immune reaction and immunodepression reaction will need to be performed.

In addition to the previous immunization experiment, infections with *G. muris* were done in order to investigate the response raised against *CWP2* in a natural infection. *G. muris* infected mice were shown to stimulate a *CWP2*-specific IgA response in the fecal pellets for up to 35 days post-infection. The detection of antibodies against *CWP2* in infected mice is interesting because it indicates that this epitope is recognized by the immune system in a natural infection. It is important to note that *G. muris* infected mice recognize a *G. lamblia* cyst protein. This observation implies that *CWP2* is a conserved epitope, found in both *G. lamblia* an *G. muris* cyst structures. This introduces further motivation to use the *G. muris* mouse model for experiments on *CWP2* and

protection in challenge infections.

In conclusion, the data presented here indicates that *CWP2* could be a potential candidate for the development of a vaccine. *CWP2* raised a S-IgA, IgA and IgG1 antibody response which are responses that could potentially stop or reduce the formation of cyst. However, if it will actually be capable of stimulating protection remains unanswered, and further challenge infections will be required.

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SUMMARY AND CONCLUSION

CWP2 was successfully expressed as a his-tag gene fusion protein and isolated from inclusion bodies. BALB/c mice were orally immunized with *CWP2*. Because humoral immunity appears to play an important role in the elimination of trophozoites from the intestine, (Brown *et al.*, 1972; Snider *et al.*, 1985) ELISA assays were performed to determine the production of antibodies stimulated by *CWP2*.

Locally, a specific S-IgA response was detected. Systemically, *CWP2* specific IgA, IgG1 and IgG2a were all detected. This suggests that both a local and a systemic immune response were raised against *CWP2* after oral immunization. The presence of IgG1 and IgG2a suggests the involvement of both a Th1 and Th2-mediated immune response. Mice immunized with only *CWP2* were able to stimulate a local immune response, whereas, mice immunized with *CWP2* + CT were able to stimulate both a local and a systemic immune response. Because cyst formation takes place in the gut, it is important that a local IgA immune response be stimulated. However, the fact that a systemic immune antibody response is raised, especially IgG, suggests that there are more chances of memory cells being stimulated. Clearance of *Giardia* cysts from the intestine has also been shown to be T cell dependant (Stevens *et al.*, 1978), especially CD4+ T helper cell dependant (Heyworth *et al.*, 1987). Levels of cytokines produced by PP, MLN and spleen cells when stimulated with Con A and *CWP2* were measured. IFN-γ and IL-4 were produced by PP and MLN cells when stimulated with Con A; however, IL-5 was not detected. In spleen cells, IFN-γ, IL-4 and IL-5 were detected when stimulated with Con A. The dichotomy of Th1 and Th2 subsets observed in IgG subclasses is supported by the cytokines which confirm a role from both the Th1 and Th2 type

immune response locally (PP and MLN) and systemically (Spleen). However, when PP, MLN and spleen cells were stimulated with *CWP2*, very low levels of IFN-γ, IL-5 and IL-4 were detected, as compared to cells stimulated with Con A. In PP and MLN cells IFN-γ and IL-4 were detected while in spleen cells, similar cytokines were produced plus IL-5. Similar results were obtained by Djamiatun and Faubert (1996) when they challenged with trophozoite antigens, PP and spleen T lymphocytes from *G. muris* infected mice or mice immunized with *G. lamblia*. They were unable to detect measurable levels of IFN-γ, IL-4 and IL-5. The most probable reason why we did not observe high levels of cytokine release when cells were stimulated with *CWP2* could be due to the fact that *CWP2* does not trigger a polyclonal activation of lymphocyte as do mitogens like Con A. This could play in our favor when developing a vaccine, considering that lymphocyte polyclonal activation has been targeted as the possible reason for the poor success of effective parasite vaccine development, even leading to immunopathology (Reina-Sam-Martin *et al.*, 2000).

Specific IgA Ab to *CWP2* were detected in fecal pellets excreted by *G. muris* infected mice. These results suggest that *CWP2* appears to be a conserved epitope, found in both *G. lamblia* and *G. muris* cyst structures. The *G. muris* mouse model could then be used for experiments with *CWP2* and protection in challenge infections.

Several possible future studies can be done to further confirm the immunogenicity of *CWP2* and its potential as a candidate for vaccine purposes against cyst formation. These include:

1) Infecting immunized mice with *G. muris* cysts and measuring cyst release to determine if *CWP2* triggered protection.

2) Immunizing mice with *CWP2* and different cytokines used as adjuvants to enhance the immune response.

3) Immunizing mice with *CWP2*, delivered through bacterial carriers such as *Streptococcus gordonii* and *Lactococcus lacti*, to determine if the immune response stimulated will be stronger than with C*WP2* alone and if it will trigger longer lasting protection.

4) Immunizing mice with *CWP1* to see if the immune response stimulated is similar to the response stimulated by *CWP2*.

5) Immunizing mice with both *CWP1* and *CWP2* to see the immune response stimulated by the combination of those cyst proteins.

6) Optimizing concentrations of *CWP2* used to stimulate a culture of cells to try to detect higher levels of cytokines.

7) Performing a complete analysis of the type of immune responses stimulated after mice have been immunized with *CWP2*. For example, does immunization with *CWP2* lead to:

-non-specific immune responses

-inflammation reaction

-auto-immune reaction

-immunodepression reaction

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