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The therapeutic action of monoclonal antibodies against a surface glycoprotein of *Giardia muris*

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SUMMARY

Twenty-three monoclonal antibodies (mAbs) against *Giardia muris* were obtained in two fusions using spleen cells of immunized mice. Similarly, the fusion of mesenteric lymph node (MLN) cells of an infected mouse yielded 15 mAbs. Three mAbs arising from spleen cells and two from MLN (all five were IgM) were able to kill trophozoites in the presence of guinea-pig complement (GPC). They can agglutinate trophozoites and also impair the movement of the flagella in the absence of GPC. I.p. treatment of mice with mAb 45C on Days −1, 1, 3 and 5 of infection significantly reduced the intestinal parasite burden. Indirect immunofluorescence assays on live and fixed trophozoites revealed that the cytotoxic mAbs were directed against antigens located on the periphery of the body, the sucking disc, the flagella and the ventro-lateral flange. In Western blots, our mAbs recognized major 36,200 and 30,300 MW glycoproteins located on the surface of the parasite.

INTRODUCTION

The *Gardia muris* mouse model, described by Roberts-Thomson *et al.* (1976), has been used extensively to study giardiasis as a model for human disease. Using this model, several studies have demonstrated the presence of anti-*Giardia* antibodies in the serum and intestinal fluids of infected mice. Differences in the levels of anti-*Giardia* serum immunoglobulins in susceptible and resistant strains of mice are difficult to demonstrate when crude antigens are used in the immunoassay. Underdown *et al.* (1981) detected specific IgA in the resistant DBA/2 and BALB/c mice, as well as in the susceptible C3H/He mouse strains, after resolution of infection. However, differences in serum antibody levels to specific *G. muris* antigens between susceptible and resistant mouse strains have been observed (Erlich *et al.*, 1983). Anders, Roberts-Thomson & Mitchell (1982) detected *G. muris*-specific IgA in the intestinal fluids of infected BALB/c and C3H/He mice. Interestingly, the resistant BALB/c mouse strain produced this anti-*Giardia* IgA earlier in infection than did the susceptible C3H/He strain. Using the indirect immunofluorescence assay (IFA), Heyworth (1986) demonstrated the binding of IgA and IgG to trophozoites in the intestine of BALB/c mice after Day 10 of infection. The elimination of serum and gut immunoglobulins in mice by injecting them from birth with goat anti-mouse IgM, resulted in chronic infection with *Giardia* (Snider *et al.*, 1985). This suggests that antibodies play an important role in the resolution of infection.

In vitro assays have also been used to evaluate the importance of antibodies in the resolution of infections with *Giardia*. Kaplan *et al.* (1985) demonstrated that the sera from rabbits immunized against whole trophozoites of *G. muris*, as well as milk from infected mice, enhanced the adherence and phagocytosis of trophozoites by murine neutrophils and macrophages. They observed the same phenomenon using the IgG fraction from rabbit sera and the IgA fraction from milk. Belosevic & Faubert (1986) observed lysis of trophozoites in the presence of sera of infected susceptible A/J and resistant B10.A mice. They also observed immobilization of trophozoites, which they defined as a lack of flagellar movement. Hill, Burge & Pearson (1984) reported that axenically cultured trophozoites of *G. lamblia* were killed by human sera that contained anti-trophozoite antibodies and GPC. In a recent study, Nash & Aggarwal (1986) demonstrated a strain-specific mAb that was cytotoxic for only the WB strain of *G. lamblia in vitro*.

To date, no study has examined the cytotoxic potential of mAbs raised against *G. muris*. The aims of this study were four-fold: (i) to examine the cytotoxicity of mAbs against trophozoites of *G. muris*; (ii) to characterize antigens of the parasite using mAbs; (iii) to determine if cytotoxic mAbs could exert a therapeutic effect on mice infected with *Giardia*; and (iv) to compare cytotoxic mAbs produced in fusions involving spleen cells with those from fusions involving mesenteric lymph node (MLN) cells.

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MATERIALS AND METHODS

Animals

Outbred female CD-1 Swiss mice were used to maintain *G. muris*, and BALB/c mice (Charles River Canada Inc., St

Constant, Quebec) were used in the production of mAbs. Prior to either infection with *G. muris* or immunization, three fecal examinations were performed to confirm the absence of *Giardia spp.* infection in these mice. Nevertheless, mice were treated for 3 consecutive days with metronidazole (CIE Neo Drug Co., Montreal, Quebec) at 10 mg/mouse/day to eliminate other protozoan infections.

The parasite

Giardia muris used in this study was originally passaged in mice by Roberts-Thomson *et al.* (1976) and was obtained from Brian J. Underdown, McMaster University, Hamilton, Ontario. The isolation of *G. muris* cysts has been described previously (Belosevic & Faubert, 1983). The inoculation dose in all experiments was 10^3 cysts per mouse, administered by gavage in 0·2 ml of phyiological saline to unanaesthetized mice. Trophozoites were isolated from the small intestines on Day 8 of infection, as described elsewhere (Belosevic & Faubert, 1983).

Preparation of antigens

A crude extract of *G. muris* antigen was obtained by sonicating trophozoites for 15 min on ice using an Ultrasonic Generator (Fisher, Montreal) at maximum output. The sonicate was centrifuged at 500 *g* for 5 min at room temperature. The supernatant was retained and the pellet discarded. The protein content was measured by the method of Bradford (1976) using bovine serum albumin (BSA; Boehringer, Mannheim, FRG) as a standard.

Immunization and fusion

Two strategies of immunization were used in the production of mAbs: (i) 50-day-old female BALB/c mice were injected (i.p.) with 1×10^7 live trophozoites of *G. muris* suspended in 0·5 ml of PBS three times at weekly intervals; (ii) mice were infected orally with *G. muris* trophozoites.

The mouse giving the highest serum titre (ELISA) against a crude extract of *Giardia* antigens was boosted i.v. with 1×10^6 trophozoites. Three days later, the mouse was killed and its spleen cells used as a source of B lymphocytes for the fusion. The mouse immunized through infection was killed on Day 16 of infection and its mesenteric lymph nodes were used as a source of antibody-producing cells.

The fusions of spleen or mesenteric lymph node cells to myeloma cells of the P3 × 63.Ag8 line (ATCC, Rockville, MD) were performed using standard procedures (Hurrell, 1982). Hybrids producing supernatants giving positive ELISA results were cloned by limiting dilution.

Ascites production

Ascites fluid containing *Giardia*-specific antibodies was obtained by injecting 10^6–10^7 hybridoma cells i.p. into mice that had been injected by the same route with pristane (Sigma, St Louis, MO) 5 days earlier. Ascites fluid was heat inactivated and partially purified by precipitation with ammonium sulphate (Anachenia, Canada) (Hudson & Hay, 1976).

Immunoaffinity chromatography

The crude soluble antigen extract of *G. muris* was immunopurified by passage through a cyanogen bromide-activated Sepharose 4B column (Pharmacia, Uppsala, Sweden). A mAb against *G. muris* (mAb 45C) was added to activated Sepharose beads in 0·1 M $NaCO_3$ buffer (pH 8·3) containing 0·5 M NaCl and incubated at room temperature for 2 hr. After washing, the beads were blocked in 0·2 M glycine (pH 8·0) for 2 hr. Ten millilitres of crude *G. muris* extract in PBS containing 2 mM phenylmethylsuphonyl fluoride (PMSF) (Sigma) were added to the column. PBS containing a final concentration of 1 M NaCl was used to remove non-specifically bound proteins from the column. HCl-glycine (pH 2·5), containing 1 M NaCl, was used to elute antigens bound specifically to the mAb. This eluate was adjusted to pH 7 with solid Tris (Sigma) and concentrated using an Amicon PM-10 filter (Amicon, Danvers, MA) with a molecular weight cut-off of 10,000.

ELISA

The ELISA was performed as described by Voller *et al.* (1976). In all cases, antigen coating was achieved by 24-hr incubation at room temperature. Crude *G. muris* antigen (10 μg/ml in PBS) was employed in ELISAs used to evaluate immunization, to detect positive clones and to determine the isotype of mAbs. Affinity-purified giardial antigens were used in ELISA (1 μg/ml protein) to detect mAb 45C in the sera of mice. The material from peaks eluted from high-pressure liquid chromatography (HPLC) columns was used as antigens (1 μg/ml protein) to determine which peaks mAb 45C react with. Horseradish peroxidase-labelled anti-mouse IgG or IgM (Bio-Can, Mississauga, Ontario) at dilutions of 1:5000 and 1:20,000, respectively, were used. The substrate was 2,2′-azino-Di-3 ethylbenzthiazoline sulphonic acid (Sigma). The plates were read on a Titertek multiskan plate (Flow Lab, Irvine, Ayrshire, U.K.) at 414 nm. Wells with absorbancss of above 0·5 were considered positive. Serum from uninfected mice or the culture medium used to grow hybridoma cells (Iscoves-modified Dulbeccos medium with 20% FCS, 10% NCTC 135 and HT) (IMDM) were used as negative controls.

Indirect immunofluorescence assay

Trophozoites of *G. muris* (10^5), fixed with 1% formalin, were dried onto slide wells at room temperature. They were then immersed in acetone for 10 min. Undiluted hybridoma culture supernatants were added to the wells at 37° for 30 min. Fluorescein-labelled anti-mouse IgM (Bio-Can) was added at a dilution of 1:50 in PBS containing 0·001% Evan's blue (Fisher, Canada).

SDS–PAGE and Western blot

SDS–PAGE was performed by the method of Laemmli (1970) with the following modifications. A Mini Protean II electrophoresis unit (Bio-Rad, Richmond, CA) was employed. Proteins were separated on a 4% stacking and 12% separating gel. Proteins of *G. muris* and molecular weight standards (Bio-Rad) were electrophoresed simultaneously.

After electrophoresis, the proteins were transferred from the gel nitrocellulose paper using the method of Towbin, Staelin & Gordon (1979). Control strips containing low molecular weight standards (Bio-Rad) and parasite proteins were stained with amido black (Fisher). The immunoblotting assay was performed by the method of Birkett *et al.* (1985) with the following modifications. Hybridoma culture supernatants or IMDM (negative control) were incubated with nitrocellulose strips. The conjugate used was peroxidase-conjugated goat anti-mouse IgM (Bio-Can) diluted 1:500.

Epitope chemistry
An ELISA assay was used to assess the chemical nature of the epitopes bound by the mAbs. After coating the plates with a crude extract of *G. muris* trophozoites, pronase (Boehringer Mannheim, FRG) or sodium metaperiodate (Sigma) were added to the wells. Pronase was diluted to 100 μg/ml in PBS and incubated for 2 h at 37°. Sodium metaperiodate was used at 0·05 M in 0·01 M sodium acetate buffer (pH 4·5) and incubated in the dark for 24 hr at 4°C. After washing to remove these reagents, the standard ELISA procedure was followed.

High-pressure liquid chromatography (HPLC)
A Bio-Gel HPHT hydroxylapatite column (Bio-Rad) was used to fractionate the cytoplasmic, membrane and cytoskeleton proteins of *G. muris*. Trophozoites (5×10^8) were lysed in distilled water containing 2 mM PMSF and centrifuged at 48,000 *g*. The supernatant (cytoplasmic proteins) was concentrated and the pellet (containing membrane and cytoskeleton proteins) was solubulized by sonication, centrifuged (48,000 *g* at 4°) and concentrated. Both cytoplasmic and membrane cytoskeleton proteins were equilibrated with 10 mM phosphate buffer during concentration. One millilitre of each protein fraction was injected onto the column using a gradient of 10 mM to 350 mM phosphate buffer (pH 6·8) during an 80-min run.

In vitro *killing assay*
The assay was done in 24-well Linbro tissue culture plates (Flow Laboratories, Toronto, Ontario). One millilitre of IMDM containing 3×10^5 trophozoites was added to each well followed by 0·25 ml of hybridoma supernatants and 0·25 ml of GPC. In control wells, IMDM replaced hybridoma culture supernatants and/or GPC. The incubation period was 1 hr at 37° with 5% CO_2. The plates were then chilled on ice for 10 min to detach trophozoites.

Staining with fluorescein diacetate (Sigma) was used to assess trophozoite viability. A fluorescein diacetate stock solution, containing 5 mg/ml in acetone, was diluted to 50 μg/ml in PBS. Ten microliters were added to 50 μl of trophozoites in suspension from the wells. After 60 seconds, the mixture was then examined using a fluorescent microscope. The live organisms stained green while the dead organisms were colourless. One-hundred trophozoites were counted and the percentage living was determined.

Effect of mAb 45C in vivo
Ten BALB/c mice were injected i.p. with mAb 45C. Ten mice were injected with ascites flued that did not react with the antigens of *Giardia* in the ELISA and five mice were untreated. The mice were injected on Days −1, +1, +3 and +5 of infection with *G. muris*. The ascites fluid containing mAb 45C and the control ascites were both ammonium sulphate precipitated before injection into mice. Each injection consisted of 900 μg of protein in a volume of 0·5 ml.

On Day 8 of infection, both experimental and control mice were killed. The small intestine was then removed, slit longitudinally and placed in PBS with 1% formalin to dislodge the trophozoites from the intestinal wall. The number of trophozoites per intestine was determined by counting four aliquots using a haemocytometer.

Statistics
Statistical analysis was performed employing the one-way ANOVA.

RESULTS

Monoclonal antibodies

Twenty-three mAbs against *G. muris* were obtained in two fusions using spleen cells of immunized mice. Similarly, the fusion utilizing MLN cells of an infected mouse yielded 15 mAbs. Three mAbs (11B, 14A and 45C) derived from spleen cells and two mAbs (43A & 66C) derived from MLN cells were observed to be 'cytotoxic' for trophozoites of *G. muris* (Table 1). All five cytotoxic mAbs were of the IgM isotype. In the presence of GPC, these mAbs killed significantly more trophozoites than did the medium or another anti-*G. muris* mAb (mAb 34C) (Table 1). mAbs 45C, 43A and 66C were more potent than mAbs 11B and 14A in killing parasites. In the absence of complement, the cytotoxic mAbs agglutinated the trophozoites and flagella.

Effect of mAb 45C *in vitro*

Mice treated with the mAb 45C had nearly three times fewer trophozoites than did mice treated with negative ascites ($P = 0{\cdot}01$) (Table 2). When antigen purified by mAb 45C was used in the ELISA, the mean OD given by the sera of the mice

Table 1. Cytotoxicity of mAbs to *G. muris* trophozoites

	% survival (mean ± SEM)	
mAb	−Complement	+Complement
Spleen		
11B	92·3 ± 2·5	23·3 ± 2·2*
14A	95·0 ± 2·2	25·3 ± 2·0*
45C	93·7 ± 2·0	3·7 ± 2·2*
34C	95·0 ± 2·2	94·0 ± 4·5
MLN		
43A	93·0 ± 3·4	7·0 ± 0·5*
66C	97·3 ± 1·8	7·3 ± 1·2*
IMDM	95·7 ± 2·4	96·9 ± 2·4

* Statistical significant ($P < 0{\cdot}01$).
Protocol as described in the Materials and Methods.

Table 2. *In vivo* curative effect of mAbs in murine giardiasis

Treatment	Mean no. of trophozoites $\times 10^7$
mAb 45C	1·24 ± 0·06*
Neg. ascites	3·67 ± 0·28
None	4·33 ± 0·85

* Statistically significant ($P < 0{\cdot}01$).
Protocol as described in the Materials and Methods.

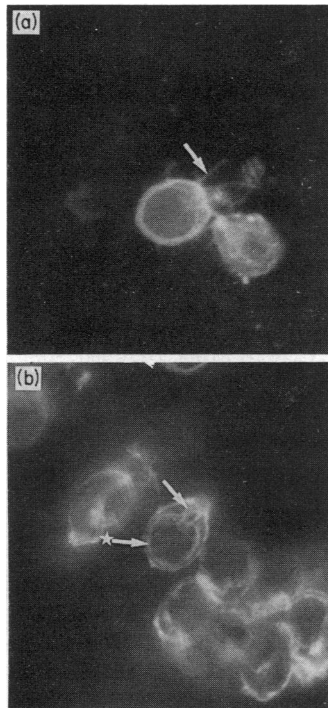

Figure 1. Indirect immunofluorescence assay indicating the antigenic targets of the five cytotoxic mAbs. (a) Flagellar fluorescence (arrow); (b) fluorescence of the outer surface, the sucking disc (arrow and star) and the ventral lateral flange (arrow).

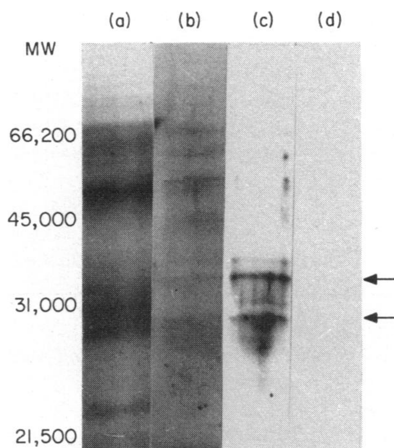

Figure 2. Western blotting of *G. muris* trophozoites. Two extracts of *G. muris* were prepared: (i) 'crude antigens' were prepared by lyophilizing trophozoites and reconstituting them in sample buffer, and (ii) the Triton-soluble extract was obtained by lysing trophozoites in a solution containing 0·5% Triton X-100 (Holberton & Ward, 1981), centrifuging at 48,700 *g* and concentrating the supernatant. (a) Crude antigen extract stained with amido black; (b) Triton-soluble antigens stained with amido black; (c) western blot reaction of cytotoxic mAb against Tritionsoluble trophozoite antigens; (d) negative control: culture supernatant incubated with and Triton-soluble trophozoite antigens. The strongest bands corresponded to Triton-soluble proteins of 36,200 and 30,300 MW (lane c: arrows).

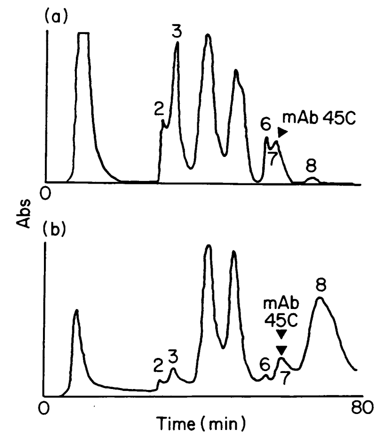

Figure 3. HPLC fractionation of membrane-cytoskeleton (a) and the cytoplasmic fractions (b). The trophozoites of *G. muris* were lysed with distilled water containing 2 mM PMSF and injected into a Bio-Gel HPHT hydroxylapatite column. All peaks were tested in the ELISA against mAb 45C.

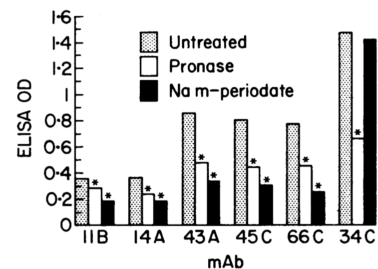

Figure 4. ELISA performed using crude trophozoite antigens that had been pre-treated with either pronase sodium metaperiodate or were untreated. These antigens were then reacted against hybridoma supernatants containing the mAbs. *Statistically significant ($P < 0·01$) from untreated.

injected with mAb 45C was higher at all dilutions and significantly higher at dilutions of 1 : 50 and 1 : 100 than that of the mice that had been treated with the negative ascites.

Specificity of mAbs and antigen location

Using the IFA, it was possible to demonstrate the specificity of the mAbs toward the trophozoite structures. The results indicate that the five cytotoxic mAbs react with the flagella (Fig. 1a), the outer edge of the sucking disk, the ventral lateral flange and the surface of the body (Fig. 1b).

Western blotting

The five cytotoxic mAbs produced the same banding pattern in the Western blot (Fig. 2). The strongest bands produced by the reaction of mAbs with membrane and cytoplasmic proteins (land c) corresponded to proteins of molecular weight of 36,200 and 30,300 (arrows). Other bands produced in lane c corresponded to proteins of 39,100 and 32,700 MW. No bands were produced when the nitrocellulose strips containing membrane and cytoplasmic proteins (land d) were incubated with IMDM as a control.

HPLC

At least eight protein peaks were obtained from HPLC of both the membrane and cytoplasmic fractions (Fig 3a, b, respectively). The similarities in elution times and the differences in absorbances of the peaks at 280 nm indicate that the two fractions contain differing amounts of the same proteins. For example peaks two, three and six are small in the chromatogram of cytoplasmic proteins (Fig 3b) but are larger in the chromatogram of membrane and cytoskeleton proteins (Fig. 3a). The opposite can be said of peak eight, which is a major component of the cytoplasmic protein fraction but not in the membrane and cytoskeleton fraction. MAb 45C reacted with peak seven only, which eluted at about 60 min from both preparations. The antigen to which mAb 45C binds is thus found in both the cytoplasmic and the membrane and cytoskeleton fractions.

Epitope chemistry

In order to determine whether or not the epitopes recognized by the mAbs contained peptide bonds, the antigen used in the ELISA was pre-incubated with the broad spectrum protease, pronase. Pronase treatment of antigen significantly lowered the amount of mAb binding to their epitopes compared to the positive controls (mAbs reacted against untreated Ag), as shown by the reduction in the ELISA OD (Fig. 4).

In order to determine if the epitopes recognized by the mAbs contained carbohydrate moieties, sodium metaperiodate (oxidizes carbohydrates) was used instead of pronase. This pretreatment with sodium metaperiodate significantly reduced the binding of the five cytotoxic mAb to their epitopes in the ELISA (Fig. 4). Interestingly, sodium metaperiodate had no significant effect on the ability of the non-cytotoxic mAb 34C to bind to its epitope (Fig. 4).

DISCUSSION

We have produced anti-*G. muris* IgM mAbs after fusion of spleen cells obtained from immunized mice or MLN cells isolated from infected mice. Five of these mAbs agglutinated trophozoites of *G. muris in vitro* and the addition of complement to the medium led to the death of the trophozoites. However, mAb 34C failed to agglutinate or kill trophozoites, in spite of the fact that the supernatant produced by this hybrid gave positive ELISA results. In fact mAb 45C has the ability to reduce the number of trophozoites in the small intestines of mice early in the acute phase of the infection. We believe that mAb 45C is probably a more potent therapeutic agent than our results show. The mice were treated with only 3·6 mg of protein via the i.p. route, which is not ideal to dislodge *Giardia* which is located in the intestinal lumen. This mAb may have gained access to the intestinal lumen due to serum leakage made possible by damage to the intestinal mucosa, which has also been observed in murine giardiasis (Gillion, Thamary & Ferguson, 1982). It is likely that the injection of mAb 45C along with an inhibitor of proteolysis intraduodenally would have been more effective.

The effect that our five cytotoxic mAbs had on trophozoites of *G. muris* in the absence of complement is of interest. The agglutination of flagella could explain the lower number of trophozoites present in the intestines of mice treated with mAb 45C. According to Holberton (1974), flagellar motion appears to be necessary for attachment to substratum. Attachment is also necessary for binary fission (Gillin & Diamond, 1981). Therefore, these agglutinins may interfere with the process of attachment and multiplication of trophozoites.

In the IFA, the cytotoxic mAbs react with the periphery of the trophozoite body, the perimeter of the sucking disk, and flagella and the ventro-lateral flange. The same pattern of fluorescence was observed with live trophozoites (i.e. not just an artifact produced by fixation), leading us to conclude that the epitopes to which these mAbs bind are located on extrinsic proteins of the parasite membrane and are not structurally altered during fixation.

The analysis of the trophozoite proteins by HPLC has demonstrated the presence of similar proteins in the cytoplasm and membrane/cytoskeleton fractions. It appears that some of the proteins exist in smaller amounts in the cytoplasm than in the membrane/cytoskeleton proteins. This might be due to the fact that the precursors of these membrane proteins may be found in the cytoplasm.

Our cytotoxic mAbs reacted most strongly with Triton-soluble proteins of 30,300 and 36,200 MW. It is possible that one of these proteins is a precursor of the other. The mAbs failed to react with cytoskeleton proteins in Western blotting (results not shown). The results of our pronase and sodium metaperiodate work leads us to assume that an epitope(s) recognized by the cytotoxic mAbs is (are) glycoprotein since pronase is a broad-specificity protease and sodium metaperiodate oxidizes carbohydrate moieties. Based on the results of the IFA, HPLC, Western blotting and epitope chemistry studies, we believe that the main targets of the cytotoxic mAbs are surface-exposed glycoproteins of 30,300 and 36,200 MW.

Membrane proteins in the 30,000 MW range have been isolated in both *G. muris* and *G. lamblia* (Crossley *et al.*, 1986; Erlich *et al.*, 1983; Taylor & Wenman, 1986). A 31,000 MW surface protein was detected in the WB strain of *G. lamblia* grown in culture (Taylor & Wenman, 1987). They observed that this was the major surface antigen of *G. lamblia* recognized during human infection and concluded that this antigen might be a disk component, giardin, described by Crossley & Holberton (1983). However, Crossley *et al.* (1986) report a 30,000 MW ventral flagellar membrane protein against which anti-giardin sera does not react. Clark & Holberton (1986) isolated five membrane proteins of *G. lamblia* that were non-iodinatable, indicating that they were intrinsic membrane (not surface exposed) proteins. In our study, weak cross-reactivity was observed by ELISA between the five cytotoxic mAbs and a crude extract of the WB strain of *G. lamblia*. This suggests that while *G. lamblia* may share this protein with *G. muris*, the epitope recognized by these mAbs is different in *G. lamblia*. Erlich *et al.* (1983) have reported that resistant BALB/c mice immunized with trophozoites of *G. muris* and challenged orally with cysts, as well as infected and challenged mice, develop serum antibodies to four acidic 32,000 MW surface glycoproteins, among others (in contrast to the susceptible C3H/He mouse strain). This antigen may be the same as one of our antigens. However, when large amounts of immune serum from infected and challenged BALB/c mice (presumably containing antibodies against this 32,000 MW antigen) were injected i.p. into infected mice of the same strain on Days 5, 7 and 9 of infection, a reduction in cyst output was not observed (Erlich *et al.*, 1983). The discrepancy between these results and our results

may be due to the fact the polyclonal serum used by Erlich *et al.* (1983) may have contained lower quantities of the cytotoxic antibodies compared to ascites fluid used in this study.

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