Serum antibodies to giardial surface antigens: lower titres in persistent than in non-persistent giardiasis

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Summary. Antisera to two antigens of Giardia lamblia—plasma membrane (PM) protein and an affinity-purified surface antigen (SA56)—were raised in rabbits, and shown to agglutinate and kill trophozoites in vitro. These antibodies were also demonstrated by ELISA in the sera of paediatric patients with giardiasis. The titres of both antibodies were significantly higher in non-persistent (acute) and asymptomatic cases than in patients with persistent infection; and the latter group did not respond to anti-giardial therapy. The inability of this group to clear G. lamblia infection, in spite of therapy, may result from the low level of antibodies which mediate the killing of trophozoites.

Introduction

Giardiasis, a flagellate protozoan infection of the small bowel, has come into prominence during the last decade because of an increasing awareness that it results in significant morbidity and loss of manpower. Its manifestations vary from asymptomatic colonisation to severe diarrhoea (Wright et al., 1977) with malabsorption (Hoskins et al., 1967). The clinical manifestations are known to be more pronounced amongst patients with immune deficiency and immunological unresponsiveness to the parasite antigens (Taylor and Wenman, 1987). Giardiasis is especially common in children and may result in failure to thrive (Burke, 1975). The mechanisms by which the host clears Giardia lamblia from the intestine are not fully understood. Current evidence suggests that both humoral and cellular immune responses are important in clearing the parasite and providing immunity to reinfection (Ament and Rubin, 1972; Roberts-Thomson and Mitchell, 1978; Stevens et al., 1978; Snider et al., 1985). Though the trophozoites of G. lamblia are a complex mosaic of antigens, the surface-associated antigens would be important for initiation of host immune responses and activation of immunological effector mechanisms. Such responses may destroy the parasites or inhibit their multiplication (Scott and Snary, 1979; Holden and Freeman, 1981). We have recently isolated and characterised a major 56-Kda surface antigen of G. lamblia trophozoites (Kumkum et al., 1988), and the present study was designed to investigate the occurrence and biological significance of antibodies to this antigen in persistent and non-persistent cases of giardiasis.

Materials and methods

Preparation of crude giardia extract

Trophozoites of G. lamblia were axenically cultured in Diamond's TPS-1 medium (Diamond, 1968). Washed trophozoites, suspended in phosphate-buffered saline (PBS) containing Triton X-100 1%, were sonicated and then centrifuged at 20 000 g for 30 min (Kumkum et al., 1988). The supernate was labelled crude giardia extract (CGE).

Preparation of plasma membrane

Plasma membrane (PM) from trophozoites of G. lamblia was isolated and its purity checked by membrane marker enzymes Ca\(^{++}\) ATPase, Mg\(^{++}\) ATPase and Na\(^{+}\) K\(^{+}\) ATPase, as described by Kumkum et al. (1988).

Identification of surface antigen SA56

Surface antigens of G. lamblia were identified by immuno-absorption and crossed immuno-electrophoresis (Kumkum et al., 1988). A major surface antigen of trophozoites was detected by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting; it had a molecular weight of 56 Kda (fig. 1) and was designated surface antigen 56 (SA56).

Preparation of rabbit antisera

Anti-plasma-membrane serum (anti-PM) was raised in rabbits by immunising them with PM containing 1 mg
of protein per injection (Kenny, 1971). Each animal received the first dose of antigen intramuscularly, emulsified in Freund’s complete adjuvant. Subsequently, booster injections at weekly intervals were given with Freund’s incomplete adjuvant, at several subcutaneous sites.

Monospecific antiserum to SA56 (anti-SA56) was raised (Alexander and Kenny, 1977) by immunising rabbits with precipitin peaks corresponding to SA56 obtained on crossed immuno-electrophoresis (CIE) plates. Immunoglobulin G (IgG) was isolated from anti-SA56 by DEAE cellulose ion exchange chromatography (Hudson and Hay, 1980) followed by ammonium sulphate precipitation (Saxena et al., 1986).

**Affinity purification of SA56**

SA56 was purified by the method of Hudson and Hay (1980). Briefly, 30 mg of anti-SA56 IgG was coupled to 3.5 ml of CNBr-activated sepharose 4B in bicarbonate buffer (0.1 M NaHCO₃, 0.5 M NaCl) at 25°C for 2 h. After extensive washing with bicarbonate buffer, uncoupled sites were blocked with ethanolamine hydrochloride. Approximately 20 mg of CGE in 1.5 ml of PBS was added to the washed immunosorbent column and left for 4 h at 4°C. After washing with PBS, the bound antigen was eluted at 4°C with 0.1 M glycine-HCl buffer, pH 2.5. The eluate was removed and immediately brought to pH 7-0 with 1 M Tris, pH 8.0, and dialysed against PBS. The antigen was concentrated on an Amicon ultrafiltration cell with a UM-10 filter.

**Paediatric patients and controls**

Children aged 4–11 years were in four groups.

**Persistent giardiasis.** There were 18 patients in whom *G. lamblia* infection persisted, in spite of repeated anti-giardial therapy for more than 2 months, with associated signs and symptoms that included diarrhoea, abdominal pain, weight loss and flatulence (Vinayak et al., 1987). No enteropathic bacteria or parasites other than *G. lamblia* were detected in any of these patients.

**Non-persistent giardiasis.** There were 15 patients who had gastrointestinal symptoms, and their stools were positive only for *G. lamblia* cysts or trophozoites. Eradication of *G. lamblia* infection along with cessation of symptoms was observed within 1–2 weeks after the completion of specific anti-giardial therapy (Vinayak et al., 1987).

**Asymptomatic cyst carriers.** There were eight children with *G. lamblia* cysts in their stools, but without any history of gastrointestinal symptoms.

**Controls.** Age- and sex-matched subjects with no history of gastrointestinal symptoms, and stools negative for *G. lamblia* cysts or trophozoites on repeated examination, constituted the control group of 15 children.

**Titration of children’s sera by ELISA**

Antibodies to PM and SA56 were evaluated by micro-ELISA. The antigen (PM or SA56), 750 ng in carbonate buffer, pH 9-6, was coated on to 96-well polystyrene plates at 4°C overnight. After three washings with PBS containing Tween 20 (PBS-Tween), 100 µl of serial two-fold dilutions of patients’ serum in PBS-Tween with bovine serum-albumin 0.5% were added and incubated for 2 h at room temperature. Plates were washed repeatedly with PBS-Tween, before the addition of 100 µl of a 1 in 4000 dilution of horseradish-peroxidase-conjugated anti-human IgG + IgA + IgM (Dakopatts, Netherlands) and incubation at 37°C for 1 h. After thorough washing, 100 µl of substrate solution (containing orthophenyl-diamine, Sigma, 0.05%, in 0.1 M citrate buffer, pH 5-0, and H₂O₂ 0.01%) was added. After 30 min in the dark at room temperature, the reaction was stopped by the addition of 25 µl of 4 N H₂SO₄, and colour density was measured at 492 nm. The titre was taken as the highest dilution of serum at which the absorbance value of the serum was three standard deviations above the mean OD value of the negative control samples. Results were expressed as log₁₀ of the titre; and the mean and SEM of the log₁₀ values were calculated.

Comparison of the titres in the four groups was by one-way analysis of variance, followed by a pair-wise multiple comparison procedure based on least significant differences. In addition, a two-way analysis of variance was used to evaluate the effects of age and sex.

**In-vitro action of rabbit antisera on trophozoites**

Trophozoites were exposed to anti-PM or anti-SA56 antibodies in the presence or absence of complement, in
specially designed chambers (Saxena et al., 1986). Briefly, 50 µl of RPMI 1640 medium containing 1 x 10⁴ trophozoites was incubated with 50 µl of anti-PM or anti-SA56 serum, with or without guinea-pig serum as a source of exogenous complement. The total volume was adjusted to 500 µl by addition of medium.

The agglutinating and cytotoxic effects of the antibodies were evaluated after 4 h at 37°C. An aggregate containing three or more parasites was considered as agglutination, though in most cases the antisera produced aggregates of more than 50 parasites. The percentage of parasites agglutinated was calculated from the counts of unagglutinated (free) parasites in control and test chambers.

In phase contrast microscopy, dead parasites lacked flagellar mobility and showed ballooning and degeneration (increased vacuolation, granulation of cytoplasm or membrane disruption); and by standard light microscopy they were observed to be stained with Eosin Y 2%. Only those parasites which fulfilled all these criteria were considered dead. Each experiment was performed in duplicate and more than 200 parasites were counted, to calculate the percentage dead.

**Results**

**ELISA titres of giardia antibodies in children’s sera**

Patients with persistent, non-persistent and asymptomatic giardiasis developed significant antibody titres (p < 0.01) to PM (F = 50.53, df = 3,52) and SA56 (F = 56.73, df = 3,52) as compared with uninfected healthy control subjects (fig. 2), though the titres of anti-PM and anti-SA56 were significantly lower (p < 0.05 and p < 0.01, respectively) in persistent giardiasis than in non-persistent cases or asymptomatic subjects. The differences in antibody levels to PM and SA56, between non-persistent cases and asymptomatic subjects, were not significant (p > 0.05). The titres were independent of the age of the subjects (F < 1, df = 3,52), and of their sex (F < 1, df = 3,52).

**Agglutination of trophozoites by rabbit antisera**

Trophozoites of *G. lamblia* that were incubated with normal rabbit serum (NRS) or PBS showed no agglutination: no clumps of three or more parasites were seen. However, exposure to anti-PM and anti-SA56 antibodies resulted in agglutination of 98.53 (SEM 3.26) % and 96.76 (SEM 1.10) % of parasites, respectively (fig. 3). Prior heat inactivation of the antisera (56°C, 30 min) did not reduce this agglutination significantly.

**Lethal effect of rabbit antisera on trophozoites**

A negligible number (<5%) of trophozoites was killed by NRS or PBS (fig. 4). Anti-PM and anti-SA56 antisera killed 32.55 (SEM 3.26) % and 28.82 (SEM 2.92) % of the parasites, respectively; but heat inactivation reduced the killing to 10.66 (SEM 2.77) % and 7.19 (SEM 2.16) %, respectively. The lethal effect was enhanced significantly by supple-
Fig. 3. Mean percentage (bar = SEM) of trophozoites agglutinated by anti-giardia rabbit sera: (1) anti-PM; (2) heat-inactivated anti-PM; (3) anti-SA56; (4) heat-inactivated anti-SA56; (5) normal rabbit serum; (6) phosphate-buffered saline.

mentation with complement (p < 0.01), increasing the killing to 97.52 (SEM 1.97) % and 92.15 (SEM 2.70) %, respectively.

**Discussion**

Giardia trophozoites colonise the small intestine by adhering to the epithelium with their suction disks, and often penetrate beyond the epithelium (Brandborg et al., 1967; Saha and Ghosh, 1977), thus coming in contact with serum factors such as antibodies and complement by which they may be killed or opsonised. Although circulating anti-giardial antibodies have been demonstrated in individuals suffering from giardiasis (Vinayak et al., 1978; Smith et al., 1981) their precise biological role has remained unclear.

Our study has demonstrated that a specific antibody response to PM and SA56 antigens occurs in giardiasis. Demonstration of antibodies in patients' sera suggests that the trophozoite antigen may have crossed the intestinal mucosa to stimulate the systemic lymphoid tissue, or that immunoglobulins produced by local antibody-producing cells may have crossed the epithelium to the systemic circulation (Schlamowitz, 1976). Comparison of groups of patients revealed low levels of PM and SA56 antibodies in persistent giardiasis, compared with non-persistent and asymptomatic cases. We believe that underlying IgA and IgG deficiencies (Vinayak et al., 1987) may explain the lower levels in persistent giardiasis. Recently, Taylor and Wenman (1987) have also reported depressed immunological responsiveness to surface antigens in immunoglobulin-deficient patients with chronic giardiasis. Earlier studies of Erlich et al. (1983) have also indicated immunological unresponsiveness to surface antigens in subsceptible C57H/He

Fig. 4. Mean percentage (bar = SEM) of trophozoites killed by anti-giardia rabbit sera: (1) anti-PM; (2) heat-inactivated anti-PM; (3) anti-PM + complement; (4) anti-SA56; (5) heat-inactivated anti-SA56; (6) anti-SA56 + complement; (7) normal rabbit serum (NRS); (8) NRS + complement; (9) phosphate buffered saline (PBS); (10) PBS + complement.
mice which exhibit chronic murine giardiasis. Thus, depressed immunological responsiveness to antigens like PM and SA56 of *G. lamblia* trophozoites may be a cause of chronicity in giardiasis.

In-vitro interactions of PM and SA56 antibodies with trophozoites demonstrated their lethal effect. A high degree of killing of trophozoites was produced by anti-giardial antibodies and complement, whereas supplementation of NRS with complement resulted in death of only 30–40% of trophozoites; this suggests a significant role for antibodies in the lethal effect. Hill et al. (1984) reported highly variable killing of trophozoites (8–76%) by normal human serum; most sera killed no more than 25%. The higher cytotoxicity of some of their samples was ascribed to an absorbable factor specific for *G. lamblia*, possibly antibody not detectable by indirect immunofluorescence.

We have demonstrated, in *vitro*, antibody-dependent complement-mediated killing of *G. lamblia*, and this may occur also in the intestine. Gut-associated lymphoid tissue may be activated by antibody released from the parasite, with subsequent transepithelial passage of immunoglobulins into the gut lumen (Brandtzeag, 1985). In addition, the presence of complement proteins synthesised by epithelial cells (Colten et al., 1968) and Peyer's patches (Lai A Fat and van Furth, 1975), as nonspecific biological amplification in the lumen, may mediate antibody-dependent killing of trophozoites in the gut.

From the present study, it appears that the varied susceptibility to giardiasis in man may be due partly to the varied capacity of susceptible hosts to mount antibody responses to PM and SA56 antigens. The inability of the patients with persistent giardiasis to clear *G. lamblia*, producing a chronic infection, may be due to the low levels of these antibodies, which appear to be essential for the killing of trophozoites.

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