

Antibodies to Cysts of *Giardia lamblia* in Primary Giardiasis and in the Absence of Giardiasis

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The significance of serum antibodies binding to cysts of *Giardia lamblia* was evaluated by indirect immunofluorescence. Titers of 10 or higher were found in 85.6% of adults, who had probably never had giardiasis. Titers in 118 adults (geometric mean, 29.9) were higher than in 35 children (16.4), and titers in women (42.3) were higher than in men (20.0). Titers in 150 patients with primary giardiasis (80.4) were higher than in control adults, but overlapping precluded serological diagnosis. Titers increased with the duration of infection. Female patients had higher titers (100.5) than did males (66.1), but men who had been infected longer than 45 days had high titers (132). Titers in second serum samples taken from 26 patients 2 weeks to 3 months after successful nitroimidazole treatment (58.1) were lower than before treatment (151.7) but higher than in 118 controls. We conclude that most people have antibodies which cross-react with *G. lamblia* but which are induced by other immunogens and that primary giardiasis induces protracted systemic antibody responses.

Giardia lamblia in the human small intestinal lumen causes giardiasis by essentially unknown pathogenetic mechanisms. Such parasitism means antigen presentation by the intestinal route, which may lead to systemic tolerance instead of an immune response (1, 2, 28). The immune response in human giardiasis has not been characterized and, because of the unique nature of the infection, cannot be assumed to simply follow the pattern used in the serological diagnosis of numerous other infectious diseases.

The earliest reports on systemic immune reactivity to *G. lamblia* antigens, complement fixation, and skin tests date back four decades (6, 31). Intensive studies have been directed to the possibility of serological diagnosis because the parasitological diagnosis of giardiasis may be false-negative due to prepatency (10), periodicity of cyst excretion (3), or other reasons. The results have been discrepant, ranging from perfect distinction to complete overlap of titers in patients and controls. This is true of various methods, such as indirect immunofluorescence (4, 8, 22-25, 32-35) and immunoglobulin G (IgG)-specific enzyme immunoassay (5, 7, 26, 34), as well as for studies with both forms of the antigen, cysts (4, 7, 22-25, 30, 35) and trophozoites (4, 8, 23, 26, 32-34). No method has been reproducibly shown to be capable of the clinically more relevant distinction between individual patients with and without giardiasis.

Geographic variations may explain apparently discrepant findings. Where giardiasis is endemic, its prevalence is highest in children, with up to all individuals acquiring the infection several times during the first years of life, and almost all cases are asymptomatic (19, 27, 36). Immunological studies face the lack of controls without past giardiasis and of cases of primary giardiasis. Furthermore, patients who are enrolled in studies because of symptomatic giardiasis are probably exceptions and not representative of the population. In Finland, giardiasis is rare in children and in adults who have not been traveling abroad (12, 14), most infections have an identifiable source and thus a known duration (10, 12-14, 18), and prospective studies have shown that most persons who acquire *G. lamblia* also have symp-

toms (9, 14). We report circulating antibody titers in persons who had probably never had giardiasis and in adult patients at various times after the acquisition of primary giardiasis.

MATERIALS AND METHODS

Patients. The basic study population consisted of young adult patients (students of universities in the Helsinki area) visiting one of 15 general practitioners at their own initiative and with a gastrointestinal complaint. They were referred to us for parasitological consultation, as described previously (18). All those excreting cysts of *G. lamblia* in the feces, associating the onset of their illness with a recent trip to Leningrad, USSR, and having never before visited a known endemic area were selected as subjects for this study. The trips had usually lasted for 3 days or less, with a maximum duration of 5 days.

The parasitological examinations (18) revealed no other pathogens, and most patients had negative stool culture results for a variety of bacterial pathogens, but we made no effort to exclude all the conceivable organisms or other reasons for the symptoms.

Serum samples were obtained from 150 patients fulfilling the criteria listed above; the patients represented 49 different travel groups. They were treated with one of several nitroimidazole regimens (11, 15-17), to which a prompt response like the one recently described in detail (17) was revealed by subsequent interviews. Many of the patients were also enrolled in therapeutic trials (11, 15-17), and serum samples taken from 26 volunteer follow-up patients between 2 weeks and 3 months after successful treatment were tested for anti-*Giardia* antibodies.

History and clinical data. The clinical parameters were recorded before antibody studies. Each patient filled out a questionnaire during the first visit to the laboratory, recording among other details his lifelong history of traveling abroad, the exact timing of the present illness, and the trip with which he associated the onset of the illness. In addition, the 150 subjects of the study were interviewed personally.

Controls. Healthy young adults were interviewed, and 118

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persons who denied a history of persistent or intermittent gastrointestinal illness and who had never been outside Scandinavia volunteered to give serum samples. Serum samples from 35 children hospitalized for appendectomy were also used; the samples were taken for reasons unrelated to this study and made available to us.

Parasitological methods. Fresh feces immediately diluted in warm Locke's solution and Formalin-preserved samples after concentration with ether were examined with a microscope as previously described (12).

Antigen. Cysts of *G. lamblia* were purified separately from the feces of five patients by the method of Moody (21), except that a mixture of Ficoll (molecular weight, 400,000; Pharmacia, Uppsala, Sweden) and Isopaque (440 mg of iodine per ml; Medica, Helsinki, Finland) with a final density of 1.078 was used and the washing and collection of cysts from the Nuclepore filter (pore size, 8 μ m; Shandon) were done three times. The final suspension in water contained 2×10^3 to 5×10^3 cysts per ml and was distributed as 40- μ l droplets on 12-spot Teflon (Du Pont Co., Wilmington, Del.)-coated microscope slides, which were allowed to dry at room temperature and stored at -20°C . Immediately before use the slides were fixed with 94% ethanol for 10 min and washed with phosphate-buffered saline (PBS; pH 7.2). All five cyst preparations gave identical results with either patient or control sera, and antigen from one patient was used throughout the study.

Antibody titration. Antigen spots were covered with 50 μ l of serum diluted in PBS (doubling dilutions, including 1:10), and the slides were incubated in a moist chamber at 37°C for 45 min. After the slides were washed with PBS, 0.1% Evans blue, and PBS, each for 10 min, the spots were covered for 30 min with 40 μ l of fluorescein isothiocyanate-conjugated sheep anti-human immunoglobulin (molar F/P ratio, 2.9; 6.5 mg of protein per ml; preferentially detects IgG but also detects IgA and IgM; lot SH 074910; National Bacteriological Laboratory, Stockholm, Sweden) diluted 1:40. After a final 30-min wash (PBS, Evans blue, and then PBS), the slides were mounted in buffered glycerol (pH 8.5). The stained slides were examined with a Zeiss standard microscope equipped with an epi-illuminator for immunofluorescence studies, as was previously described (20), excitation filter BP 455-490, dichroic mirror FT 510, emission filter LP 520, and Osram HBO 50-W high-pressure mercury lamp. Each set of tests included appropriate dilutions of a known positive control serum to define the threshold fluorescence of the last positive dilution, i.e., to minimize the influence of day-to-day technical variation. The conjugate did not cause fluorescence of cysts covered with PBS or negative control serum. The tests were done in a blind manner: the microscopist (A.M.) did not know whose serum was being examined. The median of three titers from different sets was the result for each serum. So defined, the titers were reproducible throughout the study.

Calculations. Because the titers in various study groups had a roughly log-normal distribution, the geometric mean was used as the central value; a value of 5 was assigned to titers below 10. The antilog of the standard deviation of the logarithms of the titers was given as the measure of dispersion, i.e., this coefficient can be used to obtain the 95% confidence limits by dividing and multiplying it by the geometric mean 1.96 times. Alternatively, the logarithmic mean and standard error of the mean (SEM) are given. Log titers were used in statistical analysis, and the difference between two groups of results was subjected to Student's *t* test (two tailed) (37). The paired-sample *t* test was used in

the comparisons of pretreatment and posttreatment titers (37).

RESULTS

Comparison of patients and controls. In 118 healthy young adults, who had probably never had giardiasis, the geometric mean titer of serum antibodies to *Giardia* cysts was 29.9 (Fig. 1), and the variation was relatively wide (log mean, 1.48; SEM, 0.054). In 150 patients with similar age distribution who had symptomatic, parasitologically confirmed giardiasis, the titer was 80.4 (log mean, 1.91; SEM, 0.038) (Fig. 1), which was significantly higher than that in the controls ($P < 0.0001$). In 35 children between 4 and 14 years old, the titer was 16.4 (log mean, 1.22; SEM, 0.077) (Fig. 1), which was significantly less than that in the control adults ($P < 0.02$).

Age and sex. Since the control children had lower titers than did the adults, we looked for, but did not find, an association between age and titer for the patients (Table 1). However, the sex of the patient was significant: women had higher titers (log mean, 2.00; SEM, 0.056) than did men (log mean, 1.82; SEM, 0.050) ($P < 0.02$) (Table 1). A similar

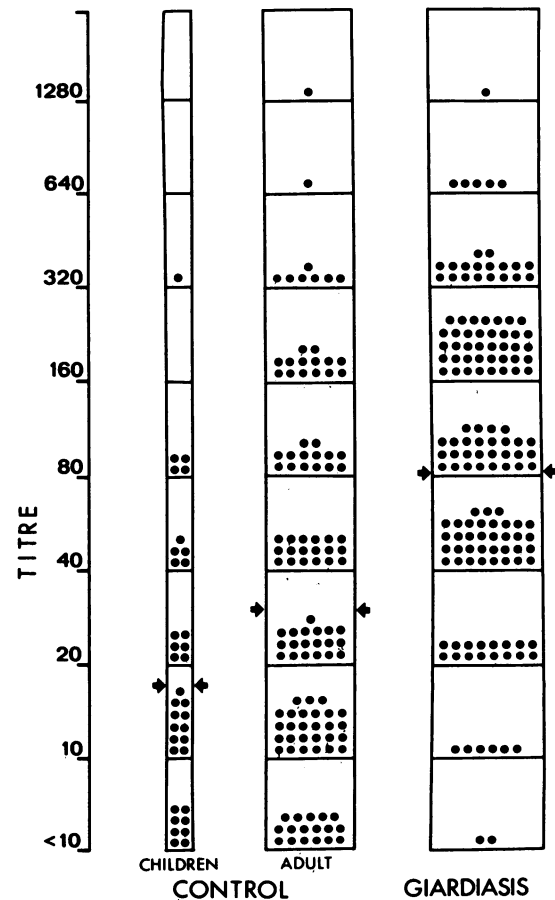


FIG. 1. Antibodies to cysts of *G. lamblia* detected by indirect immunofluorescence in the sera of 150 patients with giardiasis, 118 healthy adults, and 35 children hospitalized for appendectomy. Each dot represents one individual with the titer indicated by the line at the bottom of the box. The widths of the columns are proportional to the population sizes. The arrows indicate the geometric means.

TABLE 1. Antibodies to *Giardia* cysts in patients with giardiasis

Age (yr)	Male		Female	
	n	Titer ^a	n	Titer
15-19	15	59.3 (3.25)	9	93.3 (2.13)
20-24	36	70.6 (2.74)	39	106.3 (2.93)
25-29	22	68.3 (2.72)	18	97.0 (2.65)
≥30	7	53.8 (3.29)	4	80.0 (8.95)
All	80	66.1 (2.83)	70	100.5 (2.92)

^a Geometric mean; antilog of standard deviation of log mean is shown in parentheses.

association of sex and titer was seen in healthy adults but not in children (Table 2).

Antibodies and duration of infection. Antibodies (more than double the amount in healthy controls) were found in the patients who had acquired giardiasis 1 week earlier, and there was a positive relationship between the duration of infection and the average titer (Fig. 2). The titers in female patients were essentially unrelated to the duration of infection, whereas those in men increased with the time between the acquisition of giardiasis and obtainment of the serum sample (Fig. 2). The geometric mean titer in men before 45 days after infection was 54.1 (log mean, 1.73; SEM, 0.057); later it was 132.0 (log mean, 2.12; SEM, 0.072) ($P < 0.001$). During the first 45 days of infection women had significantly higher titers than did men ($P < 0.001$), whereas later the opposite seemed to be true.

Follow-up titers. The 26 patients from whom second serum samples were taken after successful treatment of giardiasis had an average titer of 151.7 (log mean, 2.18; SEM, 0.087) before therapy. The posttreatment titer was 58.1 (log mean, 1.76; SEM, 0.116) ($P < 0.001$), i.e., a 2.6-fold decline. The pretreatment titer in 12 male patients was 127.0 (log mean, 2.10; SEM, 0.140), the posttreatment titer was 40.0 (log mean, 1.60; SEM, 0.157) ($P < 0.001$), and the decline was 3.2-fold. The pretreatment titer in 14 female patients was 176.7 (log mean, 2.25; SEM, 0.109), the posttreatment titer was 80.0 (log mean, 1.90; SEM, 0.164) ($P < 0.002$), and the decline was 2.2-fold. Compared with the titers in 118 healthy adults (Fig. 1 and above), the posttreatment titers in the 26 patients were significantly higher ($P < 0.05$).

DISCUSSION

The results again confirmed the conclusion of other investigators that patients with giardiasis have significantly more anti-*Giardia* antibodies than do healthy individuals (4, 7, 26, 30, 32-34). The conventional interpretation is that the methods detect an antibody response induced by the giardiasis, but this causal relationship has not been formally demonstrated. Our work addressed this question at two new points.

TABLE 2. Antibodies to *Giardia* cysts in healthy adults and in children hospitalized for appendectomy

Group	Male		Female		P
	n	Titer ^a	n	Titer	
Adults	57	20.0 (3.06)	61	42.3 (4.12)	<0.002
Children	19	17.9 (2.97)	16	14.8 (2.75)	NS ^b

^a Geometric mean; antilog of standard deviation of log mean is shown in parentheses.

^b NS, Not significant ($P > 0.05$).

The titers increased with the duration of infection, and they declined after the successful treatment of giardiasis. We conclude that in our patients, giardiasis had caused a systemic antibody response detectable by cysts of *G. lamblia*.

The method of selection of the patients and controls was the essence of this investigation. In the population which they represent, the probability of finding *G. lamblia* in a person who has not recently visited a known endemic area has been repeatedly shown to be close to zero (12, 14; unpublished results). Prospective studies have shown that the acquisition of *G. lamblia* is usually followed by symptomatic giardiasis (9, 14). It is important to realize that a negative stool examination does not exclude present giardiasis (10) and tells nothing about past giardiasis. Therefore, histories of traveling and illness were used to assure that our 118 controls had probably never had giardiasis and that our 150 patients were probably experiencing their first incidence of giardiasis, with a known source and time of infection.

There is disagreement about anti-*Giardia* titers in the absence of giardiasis. With healthy controls, four groups of investigators (three using cyst antigen) who studied 85 persons found them all to be negative, with titers below 10 (4, 30, 32, 35), whereas three other groups (two using trophozoite antigen) who studied 71 persons found 18 to be positive, with titers of 16 or higher (8, 23, 33). These data indicate that anticyst titers are relatively specific for giardiasis and that the trophozoite antigens give frequent false-

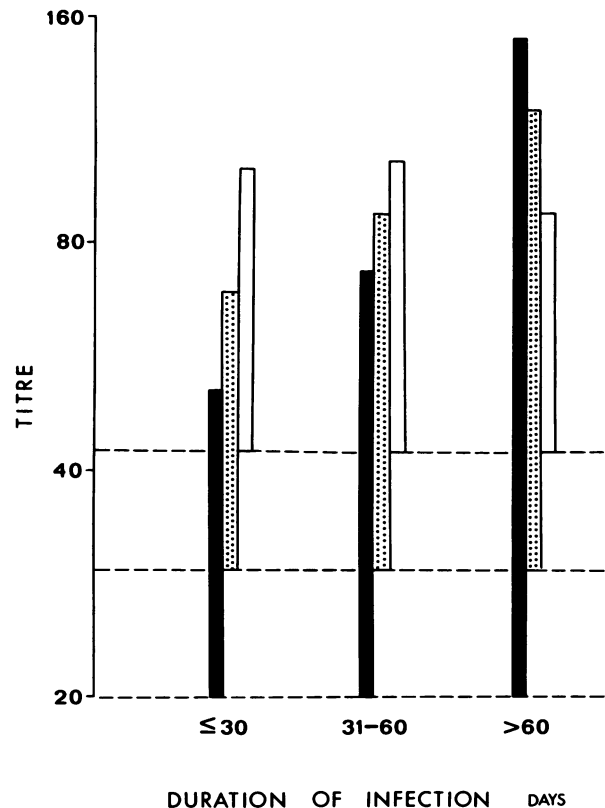


FIG. 2. Antibodies to cysts of *G. lamblia* at various intervals after the acquisition of giardiasis. Each bar shows the geometric mean titer and originates at the broken line indicating the titer in the corresponding control subjects. Symbols: ■, men (57 controls; 43 at ≤30 days, 27 at 31 to 60 days, and 10 at >60 days); □, women (61 controls; 33 at ≤30 days, 30 at 31 to 60 days, and 7 at >60 days); ▣, combined results for both sexes.

positive results. However, this impression is not entirely true, since we found similar titers with cysts in 74 of 118 controls and since most groups, including our own, have found anti-*Giardia* antibodies in various control patients without giardiasis (22, 24, 25, 32, 34, 35). Thus, different life-cycle stages of the organism do not explain the discrepancy.

The average titer in our control adults was 29.9. This could have been due to (undiagnosed) giardiasis if all 118 persons had had the disease a few weeks to a few months earlier, considering the documented rate of decline of the titer after the eradication of the disease. Assuming a 1% prevalence, although it is known to be lower (especially in the absence of a history of traveling and symptoms), and an average duration of 1 month, although it may be longer, the probability of all 118 controls having had detectable giardiasis during the preceding 6 months is less than 10^{-144} . We conclude that persons who have never had giardiasis have anti-*Giardia* antibodies and that these antibodies have been induced mainly by immunogens other than *G. lamblia*.

The use of serology for diagnosis was ruled out because the titers in individual patients with primary giardiasis were not distinguishable from those in healthy controls. Whether other methods would permit a different conclusion cannot be stated, because the relative sensitivities of the methods are not known and because previous investigators have not defined or analyzed the duration of infection or the probability of past giardiasis in their patients and controls. The latest promising attempts to find a specific serologic test for giardiasis have resorted to assay of a fraction of the interaction, i.e., to IgM instead of all the antibodies (5), or to chromatographic bands of disrupted antigen instead of whole cells (29). It remains to be seen how these methods behave in other laboratories and with other study populations.

Whereas a diagnostic test requires specificity for giardiasis, biological significance is related to all the events that are involved in the anti-*Giardia* response and to all the antibodies that bind to *G. lamblia*. The specificity of the antibodies is of no relevance: the target does not distinguish between antibodies induced by itself and those induced by other immunogens, as long as they bind to its surface; likewise, products of a true response to giardiasis might conceivably bind to a number of other (nonspecific) antigens. These are reasons to use whole-cell antigens in studies of the immune response to giardiasis. We used whole cells, and our assay obviously detected many different epitopes on the cells, i.e., a relatively broad range of different antibody specificities. Our new findings, such as the gradual increase in titer with time after infection for at least 2 months and the difference in the responses of male and female patients, which may or may not be related to the difference in the titers acquired with age in individuals without giardiasis, may or may not be detectable by methods that more selectively involve only part of the antigenic determinants or only part of the antibody specificities. Likewise, the findings may or may not be true of populations with endemic giardiasis. The findings for relatively large samples of *Giardia*-free populations and for patients with primary infections as adults must be taken into account by models of the immunobiology of human giardiasis.

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