Phenotypic and genotypic variation in *Giardia lamblia* isolates during chronic infection

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Abstract

Two *Giardia* isolates were axenised in vitro after recovery by duodenal aspiration from a man with hypo- γ globulinaemia and chronic giardiasis, before and after three unsuccessful courses of metronidazole. In vitro drug sensitivity assays showed that the pretreatment isolate was sensitive to metronidazole with minimum inhibitory concentration (MIC) and dose that inhibited growth by 50% (ED₅₀) values of 0.1 and 0.03 μ mol/l, respectively. The post-treatment isolate was 20-fold more resistant (MIC and ED₅₀ 4.3 and 0.58 μ mol/l, respectively). Differences between these isolates were also found in the surface protein profiles after radioiodination, metabolic labelling patterns with ³⁵S-methionine, malic enzyme isoenzyme patterns, and by DNA fingerprinting with a M-13 bacteriophage probe. The phenotypic and genotypic differences between the pretreatment and post-treatment isolates suggest that we have isolated two different strains from the same patient and that treatment with metronidazole resulted in selection of the more resistant strain.

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Giardiasis is the most common protozoal infection of the gastrointestinal tract. It is a frequent cause of travellers' diarrhoea and may be responsible for retarded growth and development in children. The infection is usually self limiting, being cleared within six weeks in 90% of cases. Treatment with an appropriate drug results in rapid improvement of symptoms and eradication of the parasite in 94%-100% of cases.^{1,2} Treatment failures have been documented, however, in both immunocompetent and immunodeficient patients.²⁻⁸ The mechanisms of failure of treatment are not completely understood. Some failures are probably due to inappropriate dose or failure to comply with the treatment and others to defects in the immune system of the host. Drug resistance has been proposed as another important mechanism. Differences in metronidazole sensitivity in vitro have been reported in *Giardia* isolates in axenic culture.^{9,10} In a clinical study that compared in vitro sensitivity of *Giardia* isolates to giardiacidal drugs with the clinical response to treatment, the two *Giardia* isolates with the lowest sensitivity to furazolidone were from patients who had persistent symptoms after treatment with this drug.¹¹ Furthermore, treatment with combined chemotherapy has been successful in eradicating the parasite when single drug regimens have failed.²⁻⁵ Until now all studies considering the question of drug resistance have used only one

isolate obtained either before or after treatment, thus providing limited information about the mechanisms involved in treatment failures.

We have obtained two *Giardia* isolates from a patient with hypo- γ globulinaemia and chronic giardiasis. The first was isolated before any treatment had been given and the second after three unsuccessful courses of metronidazole. We have characterised the isolates with respect to susceptibility to metronidazole and quinacrine, DNA fingerprinting, profiles of iodinated surface proteins, ³⁵S-methionine protein profiles, and isoenzyme patterns. The use of these markers for distinguishing between isolates should allow us to explore the relations between strain type, drug resistance, and treatment failure and may ultimately contribute to our understanding of the pathophysiological mechanisms involved in chronic giardiasis.

Methods

AXENISATION AND CULTURE OF GIARDIA ISOLATES
A 42 year old heterosexual man presented with a two year history of chronic diarrhoea. Faecal microscopy revealed *Giardia lamblia* cysts. Mean stool weight was increased at 500 g in 24 hours but faecal fat excretion was within normal limits at 16.6 mmol/24 h (normal range 6-18 mmol/24 h). Barium contrast radiology and endoscopy of the small and large intestine showed generalised lymphoid nodular hyperplasia that was confirmed histologically. The most significant abnormal finding was panhypo- γ globulinaemia. All serum immunoglobulin concentrations were low (IgA not detectable, IgG 5.1 (normal range 7-18) g/l, IgM 0.16 (normal range 0.4-2.5) g/l). Serum anti-*Giardia* antibodies were measured by enzyme linked immunosorbent assay (ELISA). Specific IgG (titre 1:400) was lower than would be expected in a patient with continuing infection¹² and specific IgM and IgA antibodies were not detected. Similarly, we were unable to detect specific secretory IgA in duodenal fluid. On the basis of these findings he was presumed to have chronic giardiasis, hypo- γ globulinaemia of the common variable type, and lymphoid nodular hyperplasia.

He was treated with 2 g metronidazole daily for three days with initial reduction in stool frequency and *Giardia* cyst excretion, although bowel habit never returned to normal. He received two further courses of 2 g metronidazole daily for three days on two consecutive weeks with initial improvement in stool frequency and reduction in cyst numbers. The diarrhoea returned, however, and he continued to excrete *Giardia* cysts. Duodenal aspirate confirmed the presence of *Giardia* trophozoites in the small bowel.

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Duodenal aspirates were taken at the initial clinical investigation and again three months later after three courses of metronidazole. Aspirates were transported to the laboratory on ice and the trophozoites counted by direct microscopy with a haemocytometer. Aliquots of the aspirates were centrifuged at 4°C for 10 minutes at 1000 g and the pelleted trophozoites washed once in filter sterilised, modified Diamond's TYI-S medium^{13 14} without bile, containing 10% heat inactivated newborn calf serum with penicillin (100 IU/ml), streptomycin (200 u/ml), gentamicin (50 µg/ml), and amphotericin B (0.8 µg/ml). Fresh culture medium in 16 ml borosilicate glass screw top tubes was then inoculated with 10⁶ washed trophozoites and incubated at 37°C. Trophozoites attached after several hours and the medium was changed. Growth was monitored microscopically and the medium changed every 24–48 hours. Cultures were subcultured after semiconfluent growth was seen and the antibiotics withdrawn after one to two weeks. No signs of bacterial or yeast growth were seen after this time and axenic growth was established.

DRUG SENSITIVITY ASSAY

Antigiardial activity of metronidazole and mepacrine were determined over the concentration range 0.01–1000 µmol/l as described previously.¹⁵ Briefly, cultures were initiated with 2×10⁵ trophozoites in 0.5 ml of medium from late log phase growth and incubated for 24 hours. Duplicate tubes were set up and growth assessed by counting trophozoite numbers and comparing this with that of drug free controls. Inhibition of growth was expressed as minimal inhibitory concentration (MIC), the lowest drug concentration that completely inhibited growth, and the dose that inhibited growth by 50% (ED₅₀).¹⁵

METABOLIC RADIOLABELLING WITH

³⁵S-METHIONINE

Trophozoites (2×10⁶) harvested during log growth were resuspended in 0.5 ml Diamond's TYI-S medium without serum or bile and incubated for four hours at 37°C with 20 µCi ³⁵S-methionine (Amersham International PLC, UK). After radiolabelling, trophozoites were washed twice with phosphate buffered saline (PBS) containing 0.1 mmol/l methionine, pelleted, and resuspended in sodium dodecyl sulphate (SDS) sample buffer for electrophoresis on 10% SDS-polyacrylamide gels.¹⁶ After electrophoresis at 200 V for four hours, the gel was treated with Amplify (Amersham International PLC), dried, and visualised by fluorography for 10 days at –70°C.

Sensitivities of Giardia isolates to metronidazole and mepacrine (µmol/l)

Isolate	Metronidazole		Mepacrine	
	ED ₅₀	MIC	ED ₅₀	MIC
PO1	0.56	10.0	10.0	100
B2	0.03	0.1	0.13	2.7
B3	0.58	4.3	1.8	12

Figure 1: Fluorograph of *G lamblia* strains PO1, B2, and B3 labelled with ³⁵S-methionine after electrophoresis in 10% polyacrylamide-SDS gel. Bands unique to each strain are indicated (►) and relative molecular weights calculated from size markers.

Figure 2: Isoenzyme patterns of malic enzyme analysed by isoelectric focusing of lysates from *G lamblia* strains PO1, B2, and B3. Arrows indicate individual bands, four in B2 and five in B3.

ISOENZYME ANALYSIS

Trophozoites (1×10⁷) from log phase cultures were harvested, washed three times in PBS, and lysed with 2% Triton X-100. Lysates were centrifuged at 10 000 g for five minutes and separated by electrophoresis on flat bed 1% isoelectric focusing agarose (Pharmacia) at 20 mA and 1000 V for 20 minutes. Replica gels were stained for glucose-6-phosphate dehydrogenase (G6PDH; E.C.1.1.1.49), hexokinase (Hex; E.C.2.7.1.1), and malic enzyme (ME; E.C.1.1.1.40) by standard methods.¹⁷

RADIOIODINATION OF SURFACE PROTEINS

Surface radioiodination of 2×10⁷ washed trophozoites from log growth was performed in plastic tubes coated with 100 µg iodogen (Pierce Chemicals Ltd) in 100 µl PBS pH 7 by incubation at 37°C for 10 minutes with 100 µCi Na¹²⁵I (Amersham International PLC). Labelled trophozoites were washed three times in PBS containing 10 mmol/l glucose, 10 mmol/l dithiothreitol (DTT), and 10 mmol/l sodium iodide. Pelleted trophozoites were resuspended in 100 µl lysis buffer (10 mmol/l Tris-HCL pH 7.4, 1 mmol/l MgCl₂, 0.25 mmol/l DTT, 0.25 mmol/l phenylmethylsulphonylfluoride, 0.5% Triton X-100) and vortexed every five minutes for one hour at 4°C to extract surface membrane proteins.¹⁸ Detergent extracted proteins were centrifuged at 10 000 g for 10 minutes, solubilised in equal volumes of sample buffer, and separated by electrophoresis on 10% SDS-polyacrylamide gels. Radioiodinated proteins were visualised by autoradiography at –70°C.

DNA FINGERPRINTING WITH A M-13

BACTERIOPHAGE PROBE

Total DNA was extracted from 10⁸ washed trophozoites by lysis in 1% SDS in TEN buffer

Figure 3: Autoradiograph of SDS-PAGE analysis of the surface membrane proteins after ^{125}I labelling of trophozoites of *G. lamblia* strains PO1, B2, and B3.

Figure 4: Autoradiograph showing DNA fingerprint analysis of *Sau3A* cut genomic DNA from *G. lamblia* strains B2 and B3 with the bacteriophage M13 gene III region tandem repeat sequence as a multilocus probe. Arrows denote bands at 5 and 6 Kb present in strain B3 but not in strain B2.

(50 mmol/l Tris-HCl pH 8.0, 100 mmol/l EDTA, 150 mmol/l NaCl) and digested with proteinase K (500 mg/ml) at 55°C for three hours. DNA was recovered by phenol/chloroform extraction and ethanol precipitation and redissolved in 10 mmol/l Tris-HCl, 1 mmol EDTA, pH 8.0 by standard methods.¹⁹ Purified DNA (500 ng) was digested to completion with endonucleases *Rsa*-I, *Pvu*-II, and *Sau*3A. The digest was separated by electrophoresis on 1% agarose gels, transferred to nylon filters (Hybond-N, Amersham) by Southern blotting, and UV-crosslinked. Hybridisation of filter bound DNA with ^{32}P -dCTP radiolabelled single standard M-13 DNA was performed at 60°C for 48 hours in 7% SDS, 0.263 M Na_2HPO_4 1 mmol/l EDTA at pH 7.2 after overnight prehybridisation in the same solution containing 0.1% BSA. Filters were washed twice with saline/sodium citrate (SSC) 0.1% SDS at 42°C and autoradiographed below 70°C.

Results

CULTURE AND DRUG SENSITIVITIES

Two *Giardia* isolates from duodenal aspirates were established in axenic culture: the first (B2) was obtained before treatment and the second (B3) after three unsuccessful treatments with metronidazole. Sensitivity to metronidazole and mepacrine was measured in vitro for both isolates and the laboratory strain Portland 1 (PO1). The results were expressed as ED_{50} and MIC (Table). Sensitivities to metronidazole and mepacrine were substantially lower in the B3 isolate than in the B2 isolate, but intermediate between B2 and a standard laboratory isolate, PO1. The B3 isolate was >20-fold more resistant to metronidazole than B2 with ED_{50} and >40-fold with MIC as the index.

PHENOTYPIC AND GENOTYPIC MARKERS

Radiolabelling with ^{35}S -methionine

Metabolic radiolabelling of trophozoites with ^{35}S -methionine and analysis of the labelled protein profile by SDS-PAGE revealed qualitative and quantitative differences between the isolates (Fig 1); B2 showed unique proteins of 67.5, 55 and 46 kDa molecular weight, whereas B3 had major proteins of 44.5, 58, and 71 kDa. Many common bands were seen.

Isoenzymes

Analysis of the isoelectrophoretic mobility of specific isoenzymes of B2, B3, and PO1 revealed similar patterns for the enzymes G6PDH and hexokinase (data not shown). Differences were however found in malic enzyme pattern (Fig 2). B3 showed 5 bands whereas B2 and PO1 showed the same pattern of 4 bands.

Radiolabelling of surface protein

Surface proteins of the *Giardia* isolates were analysed by SDS-PAGE and autoradiography after surface radiolabelling with ^{125}I . Incorpora-

tion of ^{125}I into the detergent soluble membrane fraction ranged between $2.0\text{--}11.0 \times 10^5$ cpm/ 10^6 trophozoites. Figure 3 shows the differences in the surface labelled proteins of the isolates and PO1. Isolate B2 had a major protein of 70 kDa with minor components at 82, 58, 31, and 22 kDa. Isolate B3 had the 70 kDa protein as a minor band with a major band at 24 kDa and a third broad band at 28 kDa. Variation in surface proteins between the isolates was thus clearly shown; five other laboratory strains showed a pattern similar to PO1 (data not shown).

DNA fingerprinting with an M-13 bacteriophage probe

M-13 hybridisation bacteriophage with all three restriction endonucleases resulted in visualisation of 10–15 bands per isolate ranging in size from 0.5 to 20 kb. Several bands were common to both isolates but, there were clear differences between B2 and B3. The *Sau* 3A cut revealed the presence of two extra bands of around 5 and 6 kb in B3 that were absent in B2. There were also minor differences with the other two cuts (Fig 4).

Discussion

During the past 10 years it has been possible to cultivate *Giardia lamblia* in vitro and methods to differentiate *Giardia* isolates by isoenzyme profiles,^{20, 21} surface proteins,^{2, 22–25} and DNA restriction fraction length polymorphism (RFLP) and DNA fingerprinting^{26–28} have been developed. With these methods we have characterised two *Giardia* isolates obtained from the same patient before and after failure to eradicate the parasite by three courses of metronidazole. The pretreatment isolate was 20 times more sensitive to metronidazole than the post-treatment isolate. Differences were also found in the ^{125}I surface protein profiles, the metabolic labelling pattern with ^{35}S -methionine, malic enzyme isoenzyme patterns, and by DNA fingerprinting. The phenotypic and genotypic non-identity of the two isolates suggest that they are in fact two different strains.

Adaptation by *Giardia* to metronidazole can be induced in vitro by exposure to sublethal doses of the drug over a period of 66 weeks; this is reversible after drug withdrawal by 22 weeks.¹⁰ The time scale over which adaptation occurred in vitro is very different from the situation of our patient in vivo, namely three periods of three days of exposure to high concentrations of drug.

Giardia lamblia isolates can change their antigenic profile in vitro and in vivo.^{22, 24–25} This could explain the changes seen in the ^{125}I surface protein profiles in our isolates but cannot account for the other phenotypic and genotypic differences. It seems highly unlikely that only spontaneous phenotypic variation within the same isolate could have occurred when so many other differences have been found.

A critical question is whether the first (B2) and second (B3) were both present at the start of the infection. Axenization of *Giardia* isolates is itself a selection process, more robust strains being presumably more likely to survive the procedure. Thus even if exhaustive cloning of the

initial isolate B2 failed to show the presence of B3, this would not exclude the possibility that B3 could have contributed to the primary infection; the assumption would be that the more abundant or more rapid growing B2 would have survived preferentially in vitro. Alternatively, but perhaps less likely, is that immediate reinfection with isolate B3 strain may have occurred either by intrafamilial contact or zoonotic infection from pets. Cyst excretion continued, however, throughout the treatment period, and screening of family members and a pet dog failed to identify other *Giardia* infections, making this explanation unlikely.

Thus our findings suggest that the mechanism of treatment failure in this case was due to selection of a *Giardia* strain with increased resistance to metronidazole from an existing heterogeneous population of *Giardia* clones with variable sensitivities in the initial infection.

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