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Giardia duodenalis: Exposure to Metronidazole Inhibits Competitive Interactions between Isolates of the Parasite In Vitro

R. C. A. Thompson, A. J. Lymbery*, D. A. Pearce, K. C. Finn, J. A. Reynoldson, and B. P. Meloni, World Health Organization Collaborating Centre for the Molecular Epidemiology of Parasitic Infections and Institute for Molecular Genetics and Animal Disease, School of Veterinary Studies, Murdoch University, Western Australia 6150, Australia; and *Western Australian Department of Agriculture, Bunbury, Western Australia 6230, Australia

ABSTRACT: The competitive interactions of genetically distinct isolates of *Giardia duodenalis* with different growth rates were studied in vitro. Electrophoretic analysis of mixed cultures showed that competition between 2 cloned isolates occurs under normal in vitro culture conditions, with faster-growing isolates outcompeting those with slower growth rates. The addition of sublethal concentrations of metronidazole to clonal mixtures in vitro prevented the competitive exclusion, which was seen in normal culture. This apparently occurred because the drug reduced the growth rate of the faster-growing but not the slower-growing clone.

Although the mechanisms of transmission are understood, the epidemiology of giardiasis is complicated by heterogeneity in the causative organism (Thompson et al., 1993; Erlandsen, 1994). Only 1 species of *Giardia, G. duodenalis* (syn. *G. intestinalis, G. lamblia*), is believed to infect humans, yet differences in virulence, pathogenicity, infectivity, growth, drug sensitivity, and antigenicity have been reported (reviewed in Thompson and Meloni, 1993). This pronounced phenotypic variability has been shown by numerous workers to be reflected by considerable genetic diversity in *G. duodenalis* isolated from humans and other mammals from many localities throughout the world (Nash et al., 1985; Meloni et al., 1988, 1989, 1995; Andrews et al., 1989; de Jonckheere et al., 1989, 1990; Homan et al., 1992; Weiss et al., 1992; Morgan et al., 1993). Further, analysis of genetic structure within and between populations of *Giardia* suggests that genetic variation arises from predominantly asexual, clonal reproduction, although occasional bouts of sexual reproduction may occur (Lymbery and Tibayrenc, 1994; Meloni et al., 1995).

Genetic diversity in *G. duodenalis* is extensive with some clones widely distributed and others localized to a particular endemic focus (Meloni et al., 1988, 1989, 1995; Homan et al., 1992; Thompson and Meloni, 1993). In endemic areas where extensive genetic heterogeneity exists within *Giardia* populations, mixed infections with more than 1 genotype (as demonstrated by Weiss et al. [1992] and Upcroft and Upcroft [1994]) are likely to occur especially under conditions where the frequency of transmission is high, as in Aboriginal communities in the north of Australia (Meloni et al., 1992, 1995). Because *Giardia* reproduces clonally, one might expect that competition between clones would limit genetic diversity within populations, as shown to be the case in a range of free-living organisms (Wilson and Hebert, 1992). The implications from in vitro culture experiments are that competition between clones of *Giardia* does occur (Mayrhofer et al., 1992; Upcroft and Upcroft, 1994) and that the clones derived from in vitro amplification of clinically derived isolates are an unrepresentative sample.

Recent studies have demonstrated that interference with clonal competition may have an important influence on the level of genetic variation in natural populations (Weider, 1992). We have suggested previously that the regular suboptimal application of chemotherapeutic regimes could be a contributing factor to the persistence of genetic heterogeneity in some populations of *G. duodenalis* by interfering with normal competitive interactions (Thompson, 1991; Thompson and Meloni, 1993; Thompson and Lymbery, 1996). For example, in Aboriginal communities in the north of Western Australia, levels of genetic diversity are as great as between isolates from throughout the state (Meloni et al., 1995). Because of noncompliance, children in Western Australian Aboriginal communities often do not receive the full course of antigiardial chemotherapy with nitroimidazole drugs. This, combined with the well documented variable sensitivity of *G. duodenalis* to these compounds (Boreham et al., 1987; Majewska et al., 1991; Farbey et al., 1995), may inhibit competitive interactions between clones of *Giardia.*

As a first step in addressing the effects of competitive interactions and the maintenance of genetic diversity within populations of *Giardia,* we have tested the following hypotheses in this study: (1) competition between genetically distinct isolates of *G. duodenalis* occurs in vitro; (2) metronidazole has differential effects on the growth rate of genetically distinct isolates of *G. duodenalis*; and (3) competitive interactions between genetically distinct isolates of *G. duodenalis* are affected by exposure to metronidazole.

The clones, P1C10, BAH44C9, BAH44C6, and BAH3C17, used in this study were derived by the method of Binz et al. (1991) from 3 isolates (Portland 1, BAH44, and BAH3) of *G. duodenalis* of human origin. All 3 isolates have been previously characterized in our laboratory and shown to be genetically distinct (Meloni et al., 1988, 1989, 1992). Trophozoites were cultured in modified BI-S-33 medium, supplemented with bovine bile, and containing 10% newborn calf serum (Meloni and Thompson, 1987) adjusted to pH 6.95; cultures were maintained in 50-mm × 13-mm (5 ml) and 126-mm × 15-mm (16 ml) borosilicate screw-capped culture tubes and 60-ml polystyrene tissue culture flasks. Tubes were filled completely with medium and incubated on a slant at 37 C. For collection of late log phase cells, 60-ml flasks were placed on ice for at least 15 min and rotated to dislodge adhering trophozoites. Cells were collected after centrifugation at 3,500 rpm for 3 min and washed twice in cold phosphate-buffered saline (PBS). Cell pellets were immediately frozen and stored at −80 C until required for electrophoresis.

Starch gel electrophoresis was used to characterize the P1, BAH44, and BAH3 isolates present in mixed cultures and to analyze the resulting populations of harvested mixtures. Using procedures and conditions previously described (Meloni et al., 1988, 1989, 1992), 2 enzyme loci that were known to be different in all 3 isolates (phosphoglucomutase [PGM] and hexokinase [HK]; Meloni et al., 1992) were chosen to analyze mixtures. In all mixing experiments, control cultures of each clone were maintained and analyzed to test the stability of the enzyme banding patterns. In all control cultures, only the patterns corresponding to the isolate with which the culture was established were detected.

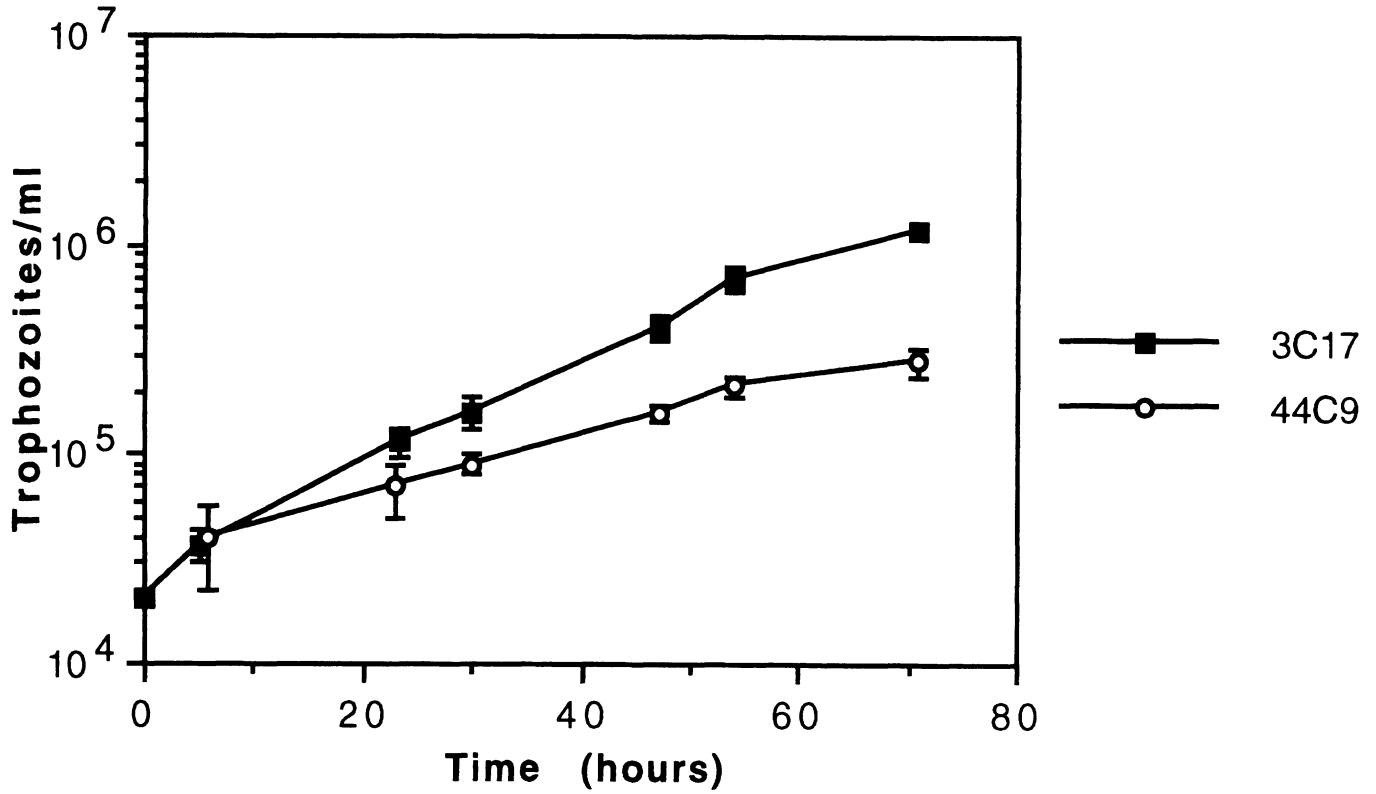

FIGURE 1. Growth curves of BAH3C17 and BAH44C9 (a), and P1C10 and BAH44C6 (b) (mean ± standard deviation at each time point).

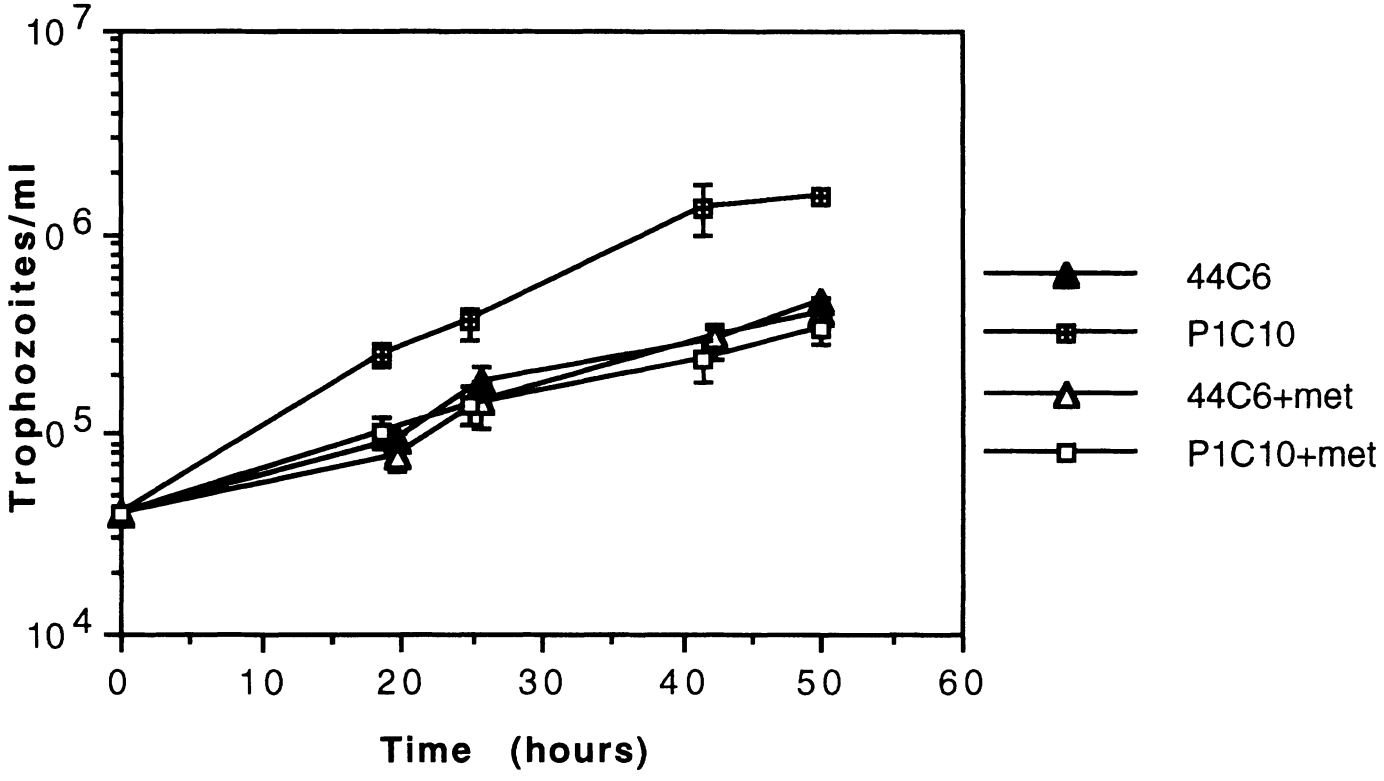

FIGURE 2. Growth of P1C10 and BAH44C6 with and without exposure to 0.2 μM metronidazole (mean ± standard deviation at each time point).

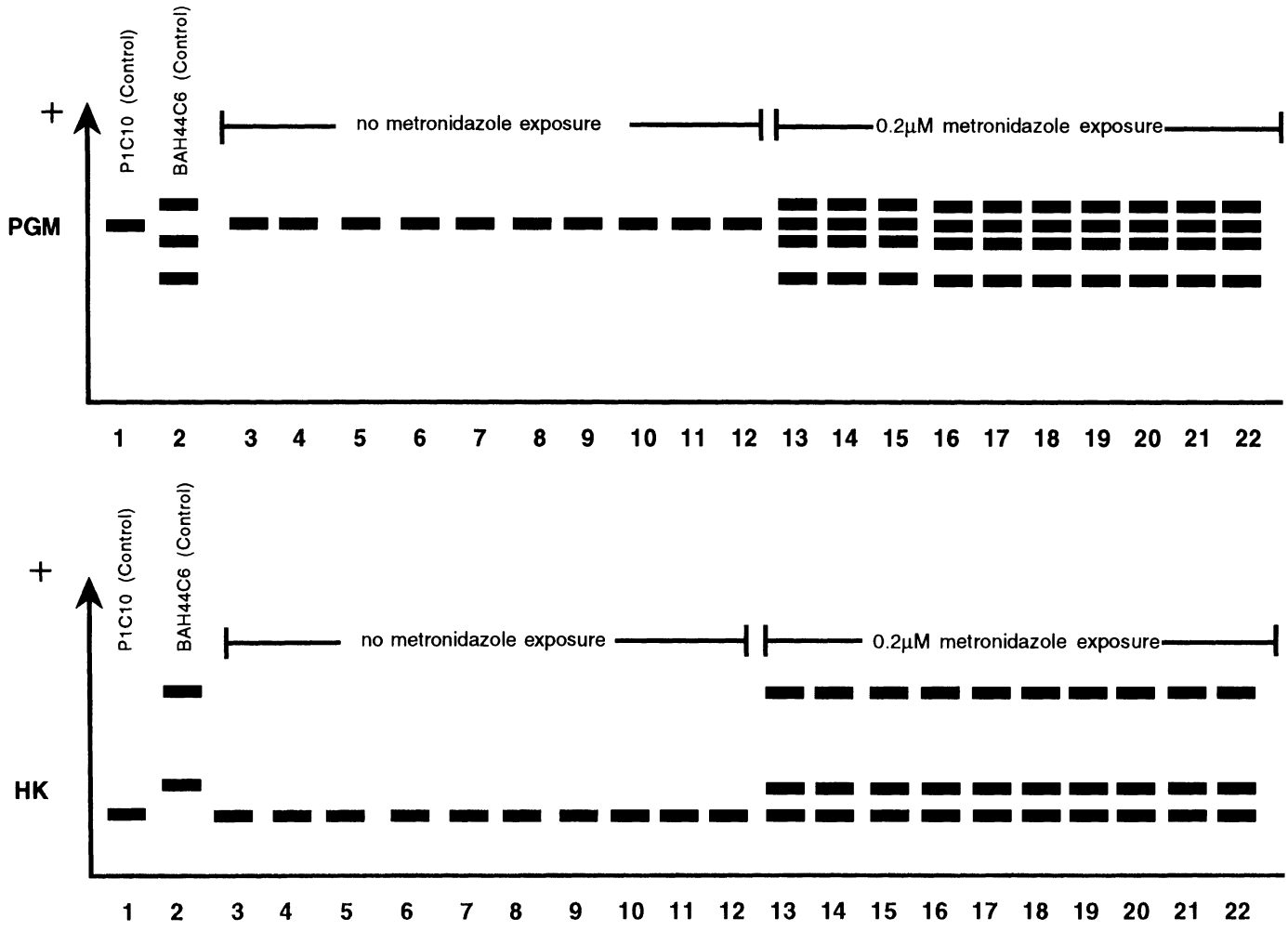

FIGURE 3. Diagrammatic representation of starch gel electrophoresis showing staining patterns of phosphoglucomutase and hexokinase of controls P1C10 and BAH44C6 and resultant cultures of mixtures of the 2 isolates with and without exposure to 0.2 mM metronidazole. Lane no. 1 represents P1C1O; lane no. 2 represents BAH44C6; lane nos. 3–12 represent 1:1 ratio of P1C1O:BAH44C6 without exposure to metronidazole; lane nos. 13–22 represent a 1:1 ratio of P1C1O:BAH44C6 with exposure to 0.2 μM metronidazole.

For each clone, 16-ml tubes were seeded with 40,000 trophozoites per ml of media, with 4 replicates for each time point. Viable cells were counted at regular intervals through the log phase using an improved Neubauer hemocytometer. To dislodge trophozoites from the wall of the culture vessels, culture tubes were placed on ice for 15 min, then rolled between the palms of the hands and inverted several times. Cultures were maintained only over the exponential part of the growth curve. The coefficient of exponential growth (r) was estimated as the slope of the line of log (cell numbers) against time in culture. Mean generation time (MGT) was estimated on the basis of doubling time.

The 4 clones exhibited different growth rates in vitro under normal conditions (Figs. 1, 2). Under the growth conditions in this experiment, for BAH3C17 MGT = 12 hr and $r = 0.025$, whereas for BAH44C9 MGT = 20 hr and $r = 0.014$ (the difference in slopes is significant, $P < 0.0001$; Fig. 1). For P1C1O MGT = 7.5 hr, $r = 0.034$, whereas for BAH44C6 MGT = 17 hr, $r = 0.022$ ($P < 0.0001$; Fig. 2). When cultured with a sublethal concentration of metronidazole (0.2 μM) the growth rate of P1C10 was reduced by 59% (with metronidazole MGT = 18.5 hr and $r = 0.018$; without metronidazole MGT = 7.5 hr, $r = 0.035$, $P < 0.0001$) while the growth rate of BAH44C6 was unaffected (MGT = 17 hr, $r = 0.022$, with and without metronidazole) (Fig. 2).

Mixed cultures of clones BAH44C9 and BAH3C17 were established in 5-ml borosilicate tubes at a seeding density of 40,000 trophozoites per ml in the proportions 1:1, 3:1, 1:3. As cultures reached monolayer stage, 1 ml was removed by 1 of 3 methods and used to establish cultures in 16-ml tubes: (1) samples of nonadhering trophozoites were subcultured from undisturbed cultures maintained at 37°C; (2) representative samples of the total trophozoite population were taken after incubating the tube on ice for 15 min and dislodging trophozoites by mixing; and (3) adhering trophozoites were sampled after all media was tipped from the tube and the tube refilled with media, incubated on ice for 15 min and trophozoites dislodged by mixing. As 16-ml cultures reached monolayer stage, 1 ml of the media was removed after ice immersion and subcultured into 60-ml flasks, as described above. Once trophozoites had attained monolayer growth in 60-ml flasks, they were collected following ice immersion.

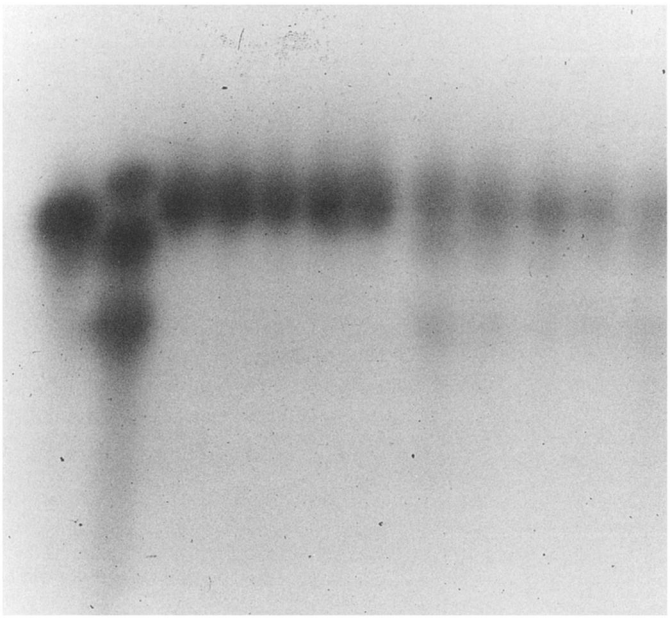

FIGURE 4. Representative starch gel showing staining patterns for phosphoglucomatase (PGM)) obtained with controls and mixtures of *Giardia* isolates P1C1O and BAH44C6, with and without exposure to metronidazole (left to right). PGM lane 1: (P1C1O control), lane 2: (BAH44C6 control), lanes 3–7: (P1C1O + BAH44C6 mixture), lanes 8–12: (P1C1O + BAH44C6 mixture with metronidazole).

A total of 50 replicate mixed cultures of isolates BAH3C17 and BAH44C9 were analyzed for the 2 isoenzymes. In 48 of these mixtures, the faster-growing isolate (BAH3C17) was the only isolate detected. Under the null hypothesis of no competition between isolates, this result is extremely unlikely to occur by chance (using the normal approximation to the binomial distribution, $Z = 16.3$, $P < 0.001$). Of the 2 exceptions, in 1 replicate (ratio 1:1, adhering trophozoites only subcultured) a mixture of the 2 isolates with BAH44C9 in higher concentrations was found, based on enzyme staining intensity after electrophoresis, whereas the other replicate (ratio 1(BAH3C17): 3(BAH44C9), total random sample of trophozoites subcultured) showed only BAH44C9. These 2 cultures took 3 days longer than comparative cultures to reach monolayer stage (12 and 13 days, respectively).

Mixed cultures of clones P1C10 and BAH44C6 were established as described above but using only equal proportions of each isolate and subculturing only by method (2). Ten replicate mixed cultures were grown in normal in vitro culture media, and 10 in media containing 0.2 μM metronidazole. In all 10 mixtures without metronidazole the faster-growing isolate (P1C10) was the only isolate detected (Figs. 3, 4), again a result inconsistent with the null hypothesis of no competition between isolates (from the binomial test, $P < 0.01$). By contrast, both isolates were detected in all 10 mixtures containing metronidazole.

These studies have shown that competition between 2 cloned isolates of *Giardia* does occur under normal in vitro culture conditions thus supporting the findings of Mayrhofer et al. (1992) and Upcroft and Upcroft (1994). In 96% (BAH3C17 vs. BAH44C9) and 100% (P1C10 vs. BAH44C6) of mixed cultures, the faster-growing isolate (BAH3C17 and P1C10, respectively) outcompeted the other. With the first experiment, this occurred even when the slower-growing isolate was seeded at 3 times the concentration of the faster-growing isolate. The concentration of trophozoites in each experimental sample was between 60 and 120 $\times$ 10^6 trophozoites per 30 μl. The electrophoretic technique employed in our laboratory is capable of detecting a minimum concentration of 3.2 $\times$ 10^6 trophozoites (data not shown), which is approximately 5% of the total sample. Therefore, whereas it cannot be assumed that the slower-growing isolates were completely absent, they constituted less than 5% of the total sample. This result was expected. The growth curves of single-clone cultures usually show a positive correlation between growth rate and maximum stationary phase concentration, suggesting that faster-growing isolates should always prevail in mixed cultures, unless there are highly asymmetric interactions between the clones (Finley and Dvorak, 1987).

The addition of sublethal concentrations of metronidazole to clonal mixtures prevented the competitive exclusion that was seen in normal culture. This apparently occurred because the drug reduced the growth rate (and presumably also the maximum stationary phase concentration) of the faster-growing (P1C10) but not the slower-growing (BAH44C6) clone. Farbey et al. (1995) found that reduced sensitivity to the effects of metronidazole is a general feature of slow-growing isolates of *Giardia*. The reasons for this difference between slow-growing and fast-growing isolates are not known and require further investigation but may be related to differences that have been found in other characteristics such as adherent ability and metabolism (Meloni et al., 1988; Binz et al., 1992; Hall et al., 1993). In this respect, a preferential effect of metronidazole on faster-growing isolates and therefore more metabolically active cells might be expected in view of the known metabolic activity of this drug (reviewed in Thompson et al., 1993). A more detailed understanding of the way in which metronidazole affects competitive interactions between clones may come by comparing the results of simulation studies with experimental data on relative clonal density at different stages of mixed culture. We are currently investigating PCR approaches to quantifying numbers of clones in culture and therefore obtaining the required data.

Although the in vitro system described here suggests that competitive interactions may be important in mixed infections with *G. duodenalis,* the situation in vivo is likely to be much more complicated. Our culture system is a very imperfect representation of the mammalian intestine and we do not know if our results can be realistically extrapolated to the field situation. Different strains of *G. duodenalis* have been shown to differ in their substrate requirements, pH preference, and region of the small intestine they inhabit (Binz et al., 1992; Hall et al., 1993; McInnes, 1994; Thompson and Lymbery, 1996), and niche segregation may reduce competitive interactions inside the host. Studies by Mayrhofer et al. (1992) have shown in a suckling mouse model that mixed genotypes of *Giardia* can coexist. Further, Hassell et al. (1994) recently showed that a mixture of coexisting genotypes may be spatially segregated even in a uniform environment, as a result of local dynamics and differences in dispersal rates. In addition, we need to consider that host factors such as immune status and/or nutrition, as well as concurrent infections (with other parasite species), may also have a significant environmental influence on competitive interac-

tions in the intestine and a direct bearing on the maintenance of clonal diversity in *Giardia* and other clonal parasites.

The hypotheses we have confirmed in this study (namely that competition occurs between clones of *G. duodenalis* in vitro, and that its effects are ameliorated by exposure to metronidazole because of differential effects of the drug on growth of the clones) are thus necessary but not sufficient conditions to support our suggestion that genetic heterogeneity in some populations of *G. duodenalis* is due to regular, suboptimal drug treatment. Our next step will be to examine the dynamics of mixed infections and drug treatment in a suitable animal model.

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