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RESEARCH BRIEF

Crithidia fasciculata: A Test For Genetic Exchange

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Index Descriptors and Abbreviations: Crithidia; Kinetoplastida; sex; drug resistance; transformation; mosquito; transmission; recombination.

Genetic exchange between cells of *Trypanosoma brucei* that occurs in the tsetse fly vector (Jenni *et al.* 1986) is an important part of the life cycle that contributes significantly to genetic diversity of these human pathogens (Gibson and Stevens 1999). Indirect observations have also indicated the presence of sexuality during the development of *Trypanosoma cruzi* (Bogliolo *et al.* 1996; Stothard *et al.* 1999) and *Trypanosoma carassii* (Zajíček 1991). However, a thorough search for this process in related *Leishmania* failed to detect the recombination process in its vector (Panton *et al.* 1991), although rare natural hybrids have been reported (Belli *et al.* 1994; Banuls *et al.* 1997). A better understanding of genetic exchange that apparently has important implications for pathogenicity and epidemiology of sleeping sickness and Chagas disease would also provide us with a novel way of combining the limited number of markers (Clayton 1999) currently available for genetic manipulations of Kinetoplastida. *Crithidia fasciculata* is a monoxenic model trypanosomatid, the Steinert and UC strains of which have existed in culture for decades. For *C. fasciculata*, a relatively high frequency of appearance of mutants with multiple resistance has been reported (Hughes *et al.* 1982). It was also observed that a mixed growth of its mutants resistant to different drugs resulted in the selection of double-resistant clones, suggesting the existence of some type of genetic exchange (Glassberg *et al.* 1985). Moreover, close apposition of the *C. fasciculata* cells during the process of flagellar adherence was reminiscent of cell fusion of other types of recombination (Hughes *et al.* 1983). To our knowledge, these results were never verified and no study is available that would search for sexuality of *Crithidia* in its mosquito host. In this work, using a highly sensitive approach, we have attempted to detect genetic exchange in this flagellate both in the insect vector and in culture.

We have used the UC strain of *C. fasciculata* genetically modified by the disruption of one allele of the single-copy ribonuclease H1 gene (*RNH1*) via targeted insertion of the neomycin (*NEO* strain) or G418 (*HYG* strain) drug-resistance cassettes as described (Ray and Hines 1995). The *RNH1* gene encodes both nuclear and mitochondrial isoforms of ribonuclease H (Engel *et al.* 2001) and seems to be nonessential, since the double knock-out strain was viable (Ray and Hines 1995). With the *C. fasciculata* strains carrying either the *NEO* or the *HYG* markers in one of their *RNH1* alleles, we had a powerful tool in our hands to search for a possible genetic cross bearing both markers in its chromosomes. Since genetic recombination in *T. brucei* has been convincingly demonstrated to occur in the vector, we have attempted to infect different mosquito species with our strains. With their decades-long cultivation in the culture form only, it was of interest to determine whether after innumerable generations the parasite did not lose its potential to develop in the insect host. Among mosquito species listed by Wallace (1966) as natural hosts of *C. fasciculata*, we were able to obtain the cultures of *Culex pipiens quinquefasciatus* and *Aedes aegypti*, and we have included *Culex pipiens molestus* in our infection experiments.

The *NEO* and *HYG* strains tolerated concentrations of G418 and

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hygromycin up to 0.25 and 0.5 mg/ml, respectively. The *NEO* strain was highly sensitive to hygromycin (lethal concentration 20 μg/ml), whereas the *HYG* strain died after the addition of 10 μg/ml of G418. Both strains were cultivated in BHI medium (Difco) containing hemin (20 μg/ml), streptomycin (100 μg/ml), penicillin (500 U/ml), and ampicillin (100 μg/ml) at 24°C. The *C. fasciculata* UC wild-type (*WT*) strain was used in control experiments. The IVth larval instars of *C. p. quinquefasciatus* (strain "H;" originating from Hyderabad, India; colonized in 1980), *C. p. molestus* (strain "M;" originating from Mosul, Iraq; colonized in 1989), *C. p. molestus* (strain "CB;" originating from České Budějovice, Czech Republic; colonized in 1986), and *A. aegypti* (an old laboratory strain without particular identification) obtained from established colonies were transferred into water containing 100 μg/ml of ampicillin to minimize bacterial contamination and were reared to adulthood. After emergence, 7- to 10-day-old adults were offered a sterile cotton pad soaked with BHI medium supplemented with 10% sucrose solution containing the *C. fasciculata NEO* and/or *HYG* strains (Table I) in which the flagellates remained alive for more than 10 h. For the following 4 to 11 days (depending on the experiment) the mosquitoes were fed with a sterile 10% sucrose solution provided in a cotton pad. After aspiration and subsequent immobilization by low temperature, the adults were immersed in 70% ethanol and washed in sterile phosphate-buffered saline (PBS), and under the dissection microscope their hindgut and rectal ampula were carefully removed. After being checked for the flagellates under light microscope, the tissue was homogenized by gentle squeezing with a cover slip, and the homogenate was than dissolved in 100 μl of sterile PBS. Two milliliters of BHI medium containing either 50 μg/ml G418 or 100 μg/ml hygromycin each were inoculated with 10 μl of the above homogenate, and 30 μl of it was added to BHI medium containing both agents. The same volumes were applied on plates with solid BHI media containing 1% agar supplemented with 70 μg/ml G418 and/or 150 μg/ml hygromycin (Table I). The inoculated media were kept for up to 10 days in an orbital shaker at 24°C and checked daily for flagellates. The agar plates were sealed with parafilm and placed into a thermostat at 28°C for up to 1 month. They were regularly checked for pinhead-sized colonies originating from a single cell.

Prior to exposure to *C. fasciculata*, all mosquito cultures used were shown both by microscopy and by cultivation to be negative for any flagellates. Next, the *C. fasciculata* strains (*NEO*, *HYG*, and *WT*) separately fed to 10-day-old adults of both sexes of the tested mosquito species were proved to be equally infective. The level of infection was different in individual mosquitoes but we have not investigated possible correlation between the flagellate and the vector species and/or sex. Heavy infections, with the intestinal contents of the host teeming with flagellates adhered to the intestinal wall, occurred in each case. Uninfected mosquitoes were then offered a pad soaked with the cultures of both the *NEO* and the *HYG* strains. After dissection, the intestinal contents of each mosquito were used for the inoculation of BHI media and agar plates containing appropriate amounts of G418 (column *NEO*), hygromycin (column *HYG*), and both G418 and hygromycin (column *NEO*/*HYG*) (Table I). The problem of bacterial contamination was largely solved by rearing of the insects in the antibiotics-containing water.

As summarized in Table I, all inoculations in liquid media with a single inhibitory agent resulted in the appearance of flagellates within 2 to 5 days. The same was true for the agar plates on which, however, colonies were spotted after 2- to 3-week-long incubation. Generally, lower numbers of colonies appeared on the G418 plates than on the hygromycin plates in the same experiment. We explain this discrepancy by the lower number of G418-resistant flagellates streaked on the plates, since these cells grow somewhat slower than the hygromycin-resistant cells. The high total number of colonies that grew on plates with a single selective agent can in fact be tripled, since only 10 μl of the intestinal homogenate was used for their inoculation compared to 30 μl applied on the plates and liquid media containing both drugs. Still, under these experimental conditions we have never observed cells with both the *NEO* and the *HYG* cassettes in their genome enabling them to grow in media containing both selection agents.

An experiment with the same design that used flagellates stably transformed with genes conferring drug resistance provided further proof for genetic exchange in *T. brucei* (Gibson and Whittington 1993). Due to the tagging of a single-copy gene, our experiments were even more sensitive than those of Gibson and Whittington, who tagged only one allele of a tubulin gene present on two or more chromosomes, and in principle we should thus be able to detect every Mendelian cross.

We have also performed a mixed growth of the *NEO* and *HYG* strains in culture. Depending on the experiment, the strains with different drug

TABLE I

Results of Mixed Growth of Two *C. fasciculata* Strains (*NEO* and *HYG*) in Natural Hosts

Mosquitoes			Liquid medium			Solid medium		
Species	Sex	Number	*NEO*	*HYG*	*NEO*/*HYG*	*NEO*	*HYG*	*NEO*/*HYG*
Aedes aegypti	M	5	+	+	−	18	72	0
	F	24	+	+	−	121	520	0
Culex p. quinquefasciatus	M	4	+	+	−	38	72	0
	F	4	+	+	−	51	124	0
Culex p. molestus "M"	M	1	+	+	−	12	28	0
	F	11	+	+	−	227	520	0
Culex p. molestus "CB"	M	5	+	+	−	159	162	0
	F	11	+	+	−	763	731	0
Total		65	+	+	−	1389	2229	0

Note. The (+) means intensive growth in the liquid medium after 5 days of incubation. Numbers in the *NEO* and *HYG* columns (solid medium) indicate number of colonies on plates after 21 days of incubation.

TABLE II
Results of Mixed Growth of Two *C. fasciculata* Strains (*NEO* and *HYG*) in Liquid Medium

Exp. No.	Days of growth	Cell density per ml	Liquid medium			Solid medium		
			NEO	*HYG*	*NEO/HYG*	*NEO*	*HYG*	*NEO/HYG*
1	3	3.1×10^6	+	+	−	160	430	0
2	4	2.5×10^7	+	+	−	1100	2100	0
3	4	3.0×10^7	+	+	−	120	330	0
4	5	1.8×10^8	+	+	−	150	380	0
5	5	2.1×10^8	+	+	−	60	150	0
Total			+	+	−	1590	3390	0

Note. The (+) means intensive growth in the liquid medium after 5 days of incubation. Numbers in the *NEO* and *HYG* columns (solid medium) indicate number of colonies on plates after 21 days of incubation.

resistance were cocultivated for 3 to 5 days and then transferred to the liquid media or plates containing both drugs (Table II). Although a high number of colonies grew on the G418- or hygromycin-containing plates, the media with both selective agents remained negative (Table II).

We did not find any support for the existence of genetic exchange in *C. fasciculata*, both in culture and in the insect host. The occurrence of such a process in cultured promastigotes of this species suggested by Glassberg *et al.* (1985) has to be considered unlikely since it was neither detected by us nor described in any flagellate studied so far (Gibson and Stevens 1999). Negative results were reported also for *Leishmania*, where the authors speculated that the sensitivity of their test may not have been sufficient to detect very rare sexual events (Panton *et al.* 1991). The stable chromosomal markers used in our experiments represent an even more sensitive tool but they failed to prove the existence of such a process in *Crithidia*. Although more data are needed, in the light of our data we propose that genetic exchange is a process absent or extremely rare in the life cycle of *C. fasciculata*.

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