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ISOLATION AND CHARACTERIZATION OF DRUG RESISTANT MUTANTS OF *CRITHIDIA FASCICULATA*

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ABSTRACT: Mutants of *Crithidia fasciculata*, resistant to Actinomycin D, Tubercidin, Crystal Violet, 6-Azauridine, and 5-Fluorouracil were obtained and characterized. The mutants were stable when maintained under nonselective conditions, and could be grouped in several subclasses on the basis of cross-resistance to other drugs. Actinomycin D- and Crystal Violet-resistant mutants appeared spontaneously whereas the others appeared only after mutagenesis with N-methyl-N'-nitro-N-nitrosoguanidine. Analysis with a cell sorter indicated no large scale meiotic changes in ploidy under the various culture conditions employed. These mutants may provide suitable, selectable, genetic markers for use in studying the possibility of a sexual mating process in *Crithidia*.

Hemoflagellate protozoa, the causal agents of many diseases of humans, animals, and even plants, are lower eukaryotic cells that are useful for studying certain basic biological problems such as mitochondrial biogenesis, the transposition and expression of chromosomal genes coding for surface proteins, and differentiation between life cycle stages. The cells have a simple genome, the complexity of which has been estimated to be from three to 15 times that of *E. coli* (Riou and Pautrizel, 1969; Klotz and Zimm, 1972; Wesley and Simpson, 1973; Leon et al., 1978; Borst et al., 1980; Castro et al., 1981; Lanar et al., 1981). They can be grown to high cell densities (> 10^8/ml) often in defined chemical media (Newton, 1956; Trager, 1957; Kidder and Dutta, 1958), and can be grown on agar (Noller, 1917; Senekije, 1944; Simpson, 1968; Wagner and Krassner, 1976; Keppel and Janovy, 1977, 1980) with plating efficiencies of 30 to 100%. However, one major drawback is the lack of the availability of a hemoflagellate genetics system.

The chromosomal number and ploidy is unknown because standard karyotyping procedures are ineffective (Vickerman and Preston, 1970), probably the result of either the lack of condensation of chromosomes at mitosis or to the small size of the chromosomes. However, there is evidence from a serial-section, electron microscopic study of log phase *Trypanosoma cruzi* culture forms (Solari, 1980) for the existence of 10 kinetochorelike regions, possibly associated with noncondensed chromosomes during mitotic division. There is also some preliminary evidence for diploidy from measurements of DNA complexity of *T. cruzi* insect forms (Castro et al., 1981; Lanar et al., 1981), *T. brucei* bloodstream forms (Borst et al., 1980), and also from the appearance of isozyme heterozygotes in bloodstream *T. brucei* cells (Tait, 1980). The latter finding also suggested the existence of a process of sexual mating. However, Amrein (1965a) was unable to detect mating of two drug resistant mutants of *T. cruzi* culture forms in the insect host. Unusual physical forms of hemoflagellates ("cyst-like bodies") have been observed in *T. conorhini* cultures (Deane and Milder, 1966, 1972), and in *T. cruzi* in the insect vector (Brener, 1972), but there is no evidence that these unusual forms are involved in a mating process and are not merely degenerative or aberrant forms.

We used the ability of *Crithidia fasciculata*, a kinetoplastid protozoan, to readily form colonies on agar and to grow in a defined medium to select and characterize drug resistant mutants. These may prove useful as selectable genetic markers to investigate the possibility of sexual mating in *Crithidia* in a systematic fashion.

MATERIALS AND METHODS

Cells

For liquid cultures cells were grown in defined KD medium (Kidder and Dutta, 1958) or Brain Heart Infusion (BHI) (Difco Laboratories, Detroit, Michigan) plus hemin (10 μg/ml), with or without gentle rotation at 27 C. The same medium could be sup-

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TABLE I. *Isolation of drug resistant mutants.*

Drug	Concentration (μg/ml)	No mutagen		Mutagenized with MNNG	
		Mutant colonies	Total cells plated	Mutant colonies	Total cells plated
Actinomycin D	10	90	1.2×10^8	39	3.6×10^7
Crystal Violet	20	41	5.0×10^7	120	3.6×10^8
6-Azauridine	100	0	2.0×10^8	5	2.6×10^7
G418	50	0	2.0×10^8	2	4.2×10^7
Tubercidin	10	0	1.8×10^8	3	2.6×10^7
5-Fluorouracil	20	0	1.3×10^8	3	1.2×10^7

plemented with 1% agar and used as a solid support for growth. Cells were spread on the agar, and plates were covered with plastic wrap to maintain moisture, inverted, and incubated at 27 C for 4 to 7 days.

Mutagenesis

Numerous compounds including antibiotics and trypanocidal drugs were screened for toxicity to wild type (wt) *Crithidia* by plating 10^7 cells on KD agar containing various concentrations of the compounds. Actinomycin D (A) (Sigma Chemical Co., St. Louis, Missouri) (10 μg/ml), Crystal Violet (CV) (Allied Chemical, Morristown, New Jersey) (20 μg/ml), 6-Azauridine (AZN) (Sigma Chemical Co.) (100 μg/ml), G418 (G) (Schering Corporation, Bloomfield, New Jersey) (50 μg/ml), Tubercidin (T) (Sigma Chemical Co.) (10 μg/ml), and 5-Fluorouracil (FU) (Calbiochem, La Jolla, California) (20 μg/ml) were lethal to wt *Crithidia* at the indicated concentrations.

Mutagenesis of cells was performed by treating a wt culture in liquid KD medium with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) (Aldrich Chemical Co., Milwaukee, Wisconsin) (2 hr, 40 μg/ml, 27 C). Stock MNNG was prepared as a 10 mg/ml solution in Hanks' buffer and stored at 4 C. Cells were then washed, cultured 18 hr at 27 C in liquid KD medium, and plated on selective KD agar at 10^7 cells per plate. The mutagenesis procedure allowed 50% survival of wild type cells as detected by plating for cell viability immediately following treatment.

Ethyl methane sulfonate (EMS) (Eastman Kodak Co., Rochester, New York) was also used. A 2-hr treatment with 12 μg/ml EMS allowed 50% survival, and after mutagenesis cells were treated exactly as in MNNG mutagenesis.

Colonies appearing on selective agar after mutagenesis (or spontaneously) were picked and streaked on KD agar containing no drug, and single colonies were picked, cultured at 27 C in liquid KD medium to log phase (approximately 8×10^6 cells/ml), and frozen in 10% glycerol at −70 and −186 C. Colonies remaining on plates were kept at 4 C and used for experiments. After 30 days these were discarded and frozen stocks of the same mutants were used to initiate new cultures.

DNA content

Cells (10^7) were fixed by pelleting in a clinical centrifuge and resuspending in 0.3% formaldehyde in 1% NaCl for 10 min. Cells were again pelleted, washed twice with NaCl, and stored at 4 C. Just before analysis, RNA was removed by treatment with 0.5 mg RNAse A (Sigma Chemical Co.) (1 mg/ml) at 37 C for 30 min. Cells were pelleted and resuspended in 0.5 ml propidium iodide (50 μg/ml) in saline and stained for 1 hr. Relative DNA content was measured by fluorescence in a Becton Dickinson fluorescence activated cell sorter (FACS) IV.

RESULTS

Selection of drug resistant mutants

The ability of *Crithidia fasciculata* to form large (2–5 mm) smooth, circular, convex colonies arising from single cells on agar containing either complex or defined medium allowed the selection of clones expressing resistance to various compounds. A wide variety of drugs were screened for toxicity to *Crithidia* both in liquid and agar media. In most cases the effective toxic concentration in agar was greater than that required in liquid medium. In addition, the effective toxic concentration in rich medium (BHI) was often greater than that required in defined medium (KD). Mutagenesis of wt cells with MNNG in liquid KD medium prior to plating on selective KD agar was performed to generate mutant strains resistant to AZN (100 μg/ml), T (10 μg/ml), and FU (20 μg/ml). Strains resistant to A (10 μg/ml) and CV (20 μg/ml) appeared spontaneously; the frequency of appearance of A and CV mutants did not increase with mutagenesis (see Table I).

Stability of drug resistant mutants

Mutants listed in Table II were tested for the stability of their drug resistance markers by growth in liquid medium in the absence of drug. At various times samples were plated on drug and drug-free agar and the relative plating efficiencies measured. All of these mutants maintained their resistant pheno-

TABLE II. *Cross-resistance of* Crithidia *mutants.*

	Strain	Drugs (μg/ml) A (10)	CV (20)	AZN (100)	G (50)	T (10)	FU (20)
a)*	A^{R1}	+†					
b)	A^{R4}	+	+				
	A^{R6}	+	+				
	A^{R14}	+	+				
	A^{R15}	+	+				
c)	A^{R5}	+			+		
	A^{R8}	+			+		
	A^{R7}	+			+		
	A^{R11}	+			+		
	A^{R12}	+			+		
a)	CV^{R2}		+				
	CV^{R13}		+				
b)	CV^{R10}		+		+		
c)	CV^{R3}	+	+	+	+		
	CV^{R9}	+	+	+	+		
d)	CV^{R9}	+	+	+		+	
a)	AZN^{R10}			+			
	AZN^{R11}			+			
b)	AZN^{R2}			+	+		
c)	AZN^{R3}			+		+	
a)	T^{R2}					+	
	T^{R10}					+	
	T^{R11}					+	
	T^{R12}					+	
	T^{R13}					+	
b)	T^{R2}				+	+	
	T^{R3}				+	+	
a)	FU^{R10}						+
	FU^{R11}						+
b)	FU^{R1}		+				+

* Strains are grouped into subclasses (a, b, etc.) showing similar cross-resistance patterns.

† + denotes that cells were able to grow on the drugs at the indicated concentrations. All other strains were either unable to grow in the presence of these drugs or were only able to grow at lower concentrations.

TABLE III. *Colony size after 7 days growth in Kidder-Dutta agar.*

Strain	With drug (mm)	Without drug (mm)
Wild type		4–5
A^{R2}	4–5	4–5
A^{R4}	4–5	4–5
A^{R5}	4–5	4–5
A^{R6}	4–5	4–5
A^{R7}	4–5	4–5
A^{R8}	4–5	4–5
A^{R11}	1–2	1–2
A^{R14}	4–5	4–5
A^{R15}	4–5	4–5
CV^{R2}	1–2	4–5
CV^{R3}	1–2	4–5
CV^{R9}	1–2	4–5
CV^{R10}	1–2	4–5
CV^{R10}	1–2	4–5
CV^{R11}	1–2	4–5
AZN^{R2}	1–2	1–2
AZN^{R3}	1–2	1–2
AZN^{R10}	1–2	4–5
AZN^{R11}	1–2	4–5
T^{R2}	1–5	4–5
T^{R3}	1–5	4–5
T^{R5}	1–5	4–5
T^{R10}	1–2	4–5
T^{R11}	1–2	4–5
T^{R12}	1–2	4–5
FU^{R1}	1–2	4–5
FU^{R10}	1–2	4–5
FU^{R11}	1–2	4–5

types when subcultured repeatedly for a total of one month in the absence of drug. In one case, where T^{R3} was examined by replica plating on drug and drug-free agar, the reversion frequency was less than 1 in 956 colonies. Mutants resistant to G418 proved to be unstable and therefore were not studied further.

Cross resistance

The mutants were tested for cross resistance to all drugs used. Cells from log phase cultures were streaked on gradient plates (Szybalski, 1952) and grown for seven days (Fig. 1). The extent of cell growth on the gradient plate indicates the level of resistance to that drug. As shown in Table II, mutants selected as resistant to a partiuclar drug can be grouped in subclasses according to their cross resistance. For example, one Actinomycin D resistant mutant (A^{R3}) was sensitive to all other drugs tested, three were also resistant to Crystal Violet, and five were also resistant to G418. Thus there appear to be at least three types of Actinomycin D resistant mutants, which may correspond to at least three different genetic loci. It is unlikely that the cells that were multiply resistant have multiple gene mutations because of the relatively high frequency of their appearance and the method in which they were obtained. Some mutants, such as CV^{R3} and CV^{R8}, which show resistance to many drugs, may be permeability mutants.

Colony size

Colony size of the mutants was examined both on drug and drug-free agar plates. Cells (10^3) from a log phase culture were spread on each plate. After 7 days, the number of colonies per plate and the size of these colonies

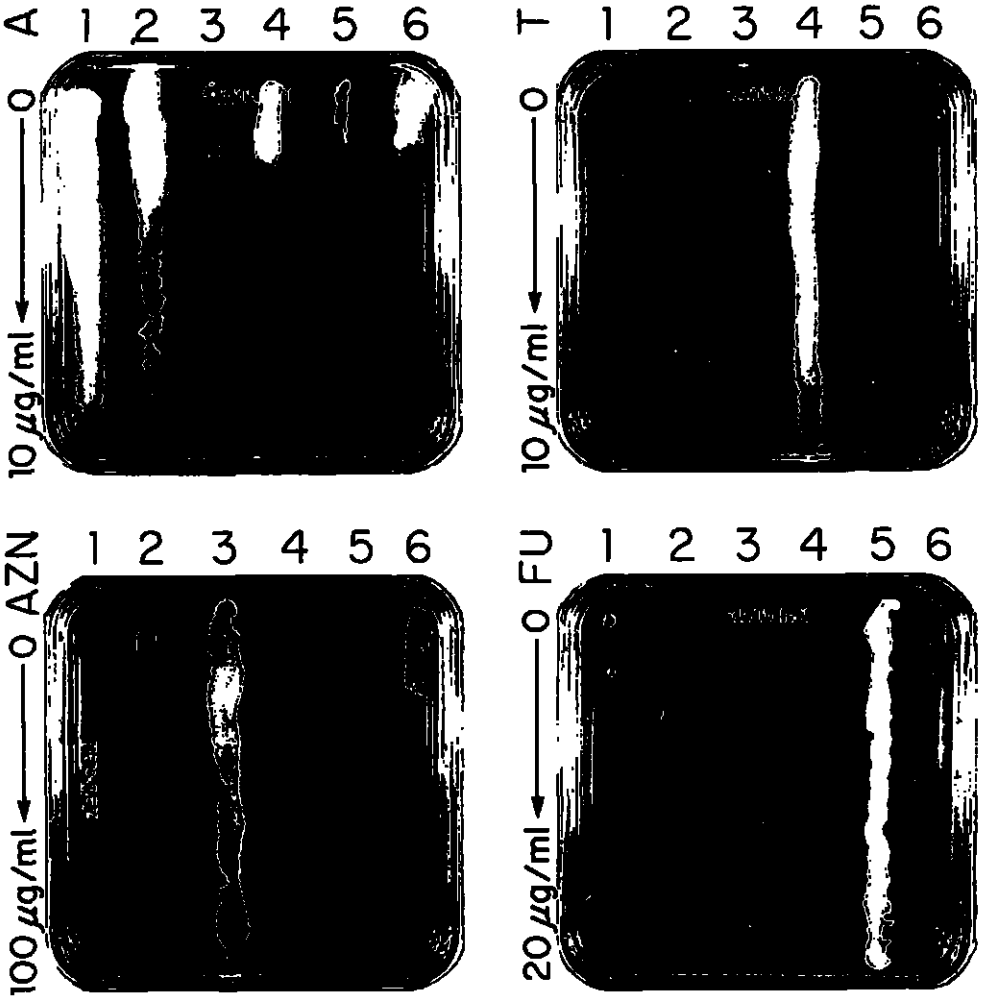

FIGURE 1. Cross resistance of mutants as demonstrated on gradient plates (Szybalski, 1952). Cells were streaked in parallel lines with a platinum loop, and plates were incubated at 27 C for 7 days; 1 = A^{R3}, 2 = CV^{R3}, 3 = AZN^{R2}, 4 = T^{R2}, 5 = FU^{R1}, 6 = Wild type.

were noted (Table III, Fig. 2). The morphology of colonies of most mutants was similar to that of wild type cells on drug-free agar. These colonies reached a diameter of 4 to 5 mm in 7 days on drug-free agar. Some of these mutants grew at the same rate on drug agar, whereas others grew slower, achieving only 1 to 2 mm in 7 days. A few mutants were slow growers on both drug and drug-free agar. All slow-growing mutants were able to continue growing after 7 days so that all the mutants tested would eventually grow to confluency on agar. Therefore, this is a measure of growth rate on agar rather than maximum colony size attainable. The growth rates of cells in liquid, both in the presence and absence of drug, were proportional to the corresponding growth rates on agar.

Most colonies arise from single cells

Because of the low plating efficiency of *Crithidia* (30–50%), the possibility exists that a minimum of two cells is required to form one colony. It is important to demonstrate that most colonies arise from single cells in order to use agar plating as a cloning technique. The percentage of doublet cells (doublets/(dou-

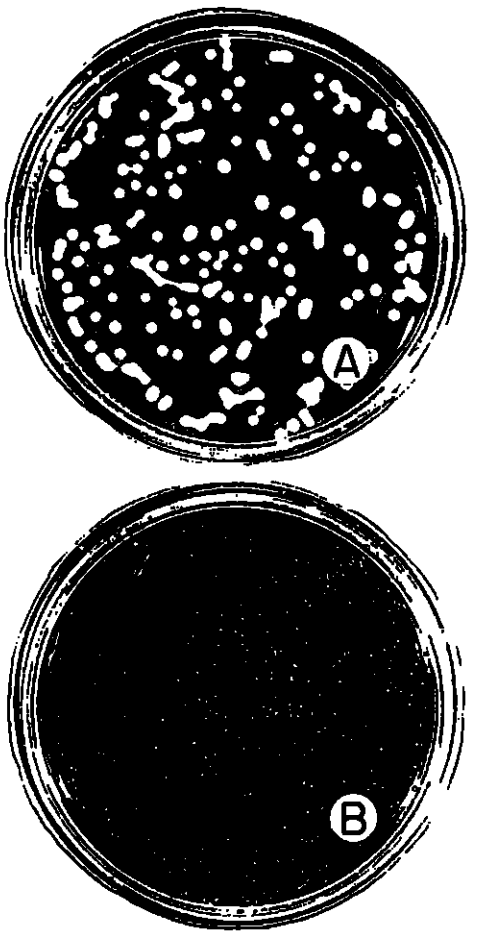

FIGURE 2. *Crithidia* colony size after seven days growth on KD drug-free agar. A. Wild type cells. B. Slow growing AZN[83].

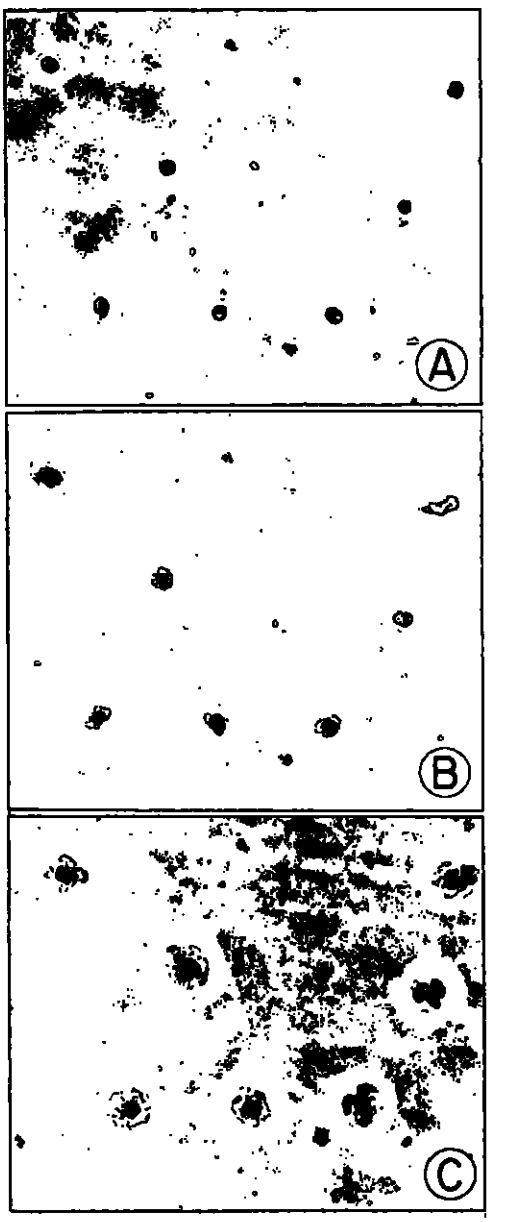

FIGURE 3. Colony growth during the first few cell divisions after plating. Mid-log phase cells were spread on KD agar and photographed through an inverted microscope, using phase contrast optics, at 0 hr (A), 8 hr (B), and 24 hr (C) after plating.

blets + singles) × 100) presumably arising from the normal process of cell division was measured in several liquid wt cultures at intervals during the growth curves. It reached a maximum of 5 to 11% in early log phase and then decreased to less than 3% in mid-log phase. The percentage of doublets usually remained low in stationary phase cultures, but infrequently, for undetermined reasons, rose to a maximum of 33%.

In order to determine whether plating on agar induces cell aggregation and whether single cells can give rise to colonies, the distribution of single and doublet cells on agar was directly monitored by phase contrast microscopy for the first few cell divisions as shown in Figure 3. In this experiment, immediately after spreading mid-log phase cells on agar, 4% of the cells appeared as doublets, which was equivalent to the frequency of doublets found in the liquid culture used for inoculation. It is clear from Figure 3 that cell–cell aggregation was not induced by spread-

ing cells on agar and that single cells did divide and gave rise to microcolonies and presumably macrocolonies. It is interesting that the microcolonies do not all grow at the same rate.

DNA content

The high frequency of appearance of the mutant phenotypes, as shown in Table I, suggests at least a transient haploid phase for these cells. To determine whether *Crithidia* have a major reduction in ploidy during the normal growth curve or under different physiological conditions, the DNA content of wt cells was measured in a FACS cell sorter. Cells were cultured under various conditions, and samples were withdrawn at different stages of the growth curve. Cells were fixed and stained as described in Materials and Methods. Two peaks appeared in all samples, presumably G_1-S-G_2 (see Fig. 4). There was no indication of a substantial third peak that would have arisen from large-scale, meiotic ploidy changes in the population.

Attempts to observe mitotic chromosomes in *Crithidia*

In an attempt to observe chromosomes in *C. fasciculata*, log phase cells were stained with 4'-6'-diamidino-2-phenylindole dihydrochloride (DAPI) and observed in the fluorescent microscope. Heterogeneous chromatin distributions were frequently observed, but no recognizable chromosomes were seen. In addition, dividing cells were processed by standard karyotyping procedures (Darlington and LaCour, 1975), and no recognizable mitotic chromosomes were observed.

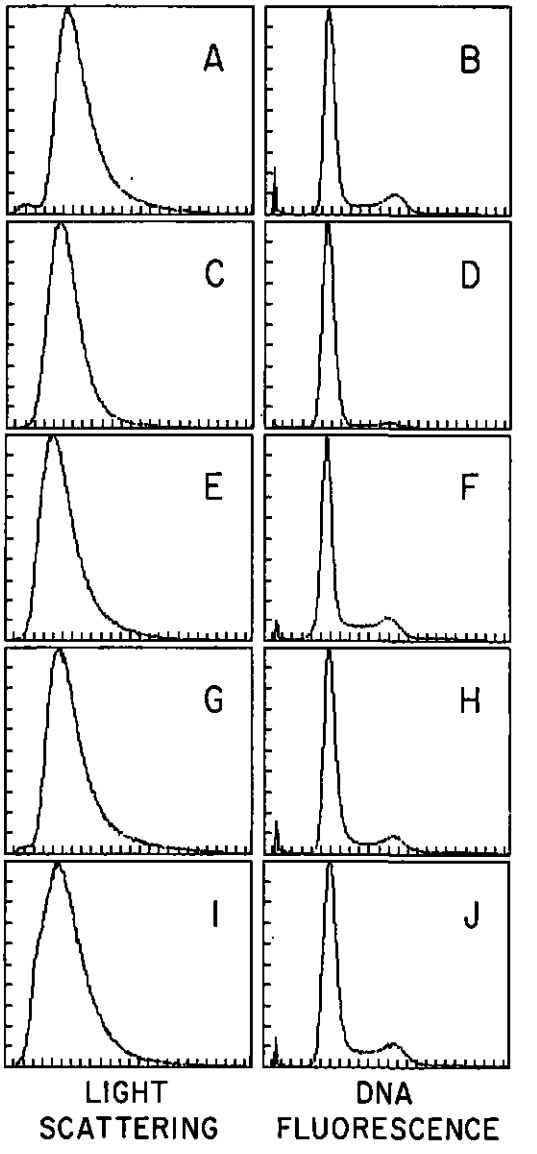

FIGURE 4. Analysis of cells on the basis of DNA content. Cells were fixed, treated to remove RNA, stained with propidium iodide, and analyzed in a FACS cell sorter. The graphs show number of cells versus light scattering, which is proportional to cell size, and number of cells versus DNA fluorescence, which is proportional to DNA content. **A** and **B**. Late log phase cells grown with high aeration. **C** and **D**. Stationary phase cells grown with high aeration. **E** and **F**. Late log phase cells grown without aeration. **G** and **H**. Stationary cells grown without aeration. **I** and **J**. Cells grown on agar.

DISCUSSION

We have isolated and characterized a number of drug resistant mutants of *C. fasciculata*. These mutants were generated by a single-step, selective agar, cloning procedure after either spontaneous appearance or induced mutagenesis. Therefore, they should be caused by single, point mutations or deletions. Previously several workers (Inoki and Matsushiro, 1959; Amrein, 1965a, b; Bacchi et al., 1974, 1975a, b) isolated drug resistant mutants of hemoflagellates by continuous adaptation to increasing concentrations of a drug. This pro-

cedure may produce mutations in several genes, the sum of which confer the final phenotype. These mutants would be poor markers when looking for possible recombination in *Crithidia* because transfer of one mutation might not be detectable. Mutants generated from single mutations, as described in this paper, would be preferable.

Drug resistant mutations can be of several different classes. They may cause inactivation of the drug, they may allow an alternative pathway for a process normally inhibited by a drug, or they may decrease permeability to the drug at the cell surface. Of the last class of mutants there are two subtypes: (a) cells may become impermeable to one particular drug caused by an alteration in the plasma membrane receptors for the drug, or (b) cells may become impermeable to a number of drugs resulting from a generalized decrease in transport across the cell membrane. A few of our mutants may be of the latter type, as they show resistance to four or five drugs tested. Jeffrey Glassberg (pers. comm.) has isolated some drug resistant mutants of *C. fasciculata* that appear to fall into the former subclass. They show decreased uptake of only one radioactively labelled drug. We have not tested our mutants in this manner.

Drug resistant mutations can be recessive, additive, or dominant (Kalow, 1962). The rate of appearance of recessive mutations will be far below the actual rate of gene mutation because of the requirement for a homozygous state for the expression of resistance. Additive mutations, as well as dominants, will be expressed at a rate similar to their mutation rates. Among the drugs tested in our studies, the expression frequencies for resistance vary, and this variation may be caused by this phenomenon of mutation expression rate. But the relatively high frequency of appearance of all of the mutants obtained in our study implies either that the markers are dominant in a diploid cell or the cell is haploid at least transiently. The cell sorter results imply an absence of any large-scale transformations of meiotic ploidy under the conditions examined, but this method would not pick up transient or infrequent changes of ploidy.

Because of the complexity of drug resistant mutants, auxotrophs may prove to be more suitable markers for use in mating studies. Nevertheless, drug resistant mutants, derived as described in this paper, may provide suitable markers in searching for a possible sexual recombination process in *Crithidia fasciculata*.

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