ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Aug. 1998, p. 2144–2145 Vol. 42, No. 8
0066-4804/98/$04.00+0
Copyright © 1998, American Society for Microbiology. All Rights Reserved.

Confirmatory Evidence from 18S rRNA Gene Analysis for In Vivo Development of Propamidine Resistance in a Temporal Series of *Acanthamoeba* Ocular Isolates from a Patient

DOLENA R. LEDEE,[1]† DAVID V. SEAL,[2] AND THOMAS J. BYERS[1]*

Department of Molecular Genetics, The Ohio State University, Columbus, Ohio 43210,[1] and Tennent Institute of Ophthalmology, Western Infirmary, Glasgow University, Glasgow, United Kingdom[2]

Received 10 April 1998/Returned for modification 5 May 1998/Accepted 10 June 1998

DNA sequences of three 18S rRNA gene alleles present in trophozoites obtained before and after therapy for *Acanthamoeba* keratitis substantiate a previous report that the infection was due to a single *Acanthamoeba* strain. Thus, the possibility that propamidine resistance which developed during therapy was due to a mixed infection was ruled out.

Acanthamoeba keratitis is a chronic progressive infection that has been increasingly identified worldwide. The condition currently is successfully managed medically in most patients (8), but it can progress to perforation of the corneal ulcer if untreated or mismanaged medically.

Many drugs have been suggested to have in vitro activity against *Acanthamoeba* spp. (5). The first successful medical cure involved topical therapy with a combination of the aromatic diamidine propamidine isethionate (Brolene) and neomycin (11). Subsequent studies confirmed the efficacy of this treatment (1), but it later was found to be successful in only half of the patients. One possible explanation for the failures was found in the observation that resistance to propamidine developed in a temporal series of *Acanthamoeba* isolates from a patient (2).

The patient had been wearing soft contact lenses and presented with a 3-week history of bilateral keratitis. Culture of corneal scrapes from each eye, with incubation at 37°C, yielded prolific numbers of *Acanthamoeba* organisms. Topical administration was initiated with the previously successful combination of propamidine (0.1% [wt/vol]) and neomycin (0.5% [wt/vol]) (11). Neomycin was withdrawn because of contact hypersensitivity. This led to recrudescence of the infection in both eyes, with *Acanthamoeba* being isolated and cultured at 37°C (2). Treatment continued with propamidine and with an arsenical (R6/56, an atoxyl derivative), but no control of the infection was achieved, and fulminant keratitis resulted. Resistance to the diamidine was associated with reduction in the temperature at which optimum growth and replication occurred (5). Further isolates at this stage would not replicate at 37°C, but did at 25°C; these isolates proved to be resistant to both propamidine and the arsenical. The infection eventually was halted by surgery on both eyes with repeated, bilateral corneal grafts (2).

The present study addresses the question of whether the infection in this patient and the subsequent development of propamidine resistance during therapy was associated with a single *Acanthamoeba* strain, or whether it resulted from a mixed infection that included both propamidine-sensitive and -resistant strains. The answer to the question depends on the experimental ability to distinguish different strains. The question was investigated previously by Kilvington et al. (6). This group compared restriction fragment length polymorphisms of mitochondrial DNA (mtRFLP) from *Acanthamoeba* obtained from the patient before therapy (isolate AcPHL/7a) and after therapy (isolates AcPHL/7b and -7c). Because the mtRFLPs for the three isolates were identical, the authors concluded that the propamidine resistance developed in a single strain due to the therapy. However, identical mtRFLPs often are found in different *Acanthamoeba* strains. For example, in three studies, samples of 8, 13, and 33 strains had only 4, 7, and 11 different RFLP phenotypes (4, 6, 12). Thus, it was possible that the RFLP analysis failed to identify the sensitive and resistant isolates as different strains. Therefore, we reexamined the issue by using a test with greater strain specificity.

The genes coding for 18S rRNA (18S rDNA) of *Acanthamoeba* have relatively high interstrain variation in DNA sequences (3, 9). In addition, strains can differ in the number of 18S rDNA alleles and the presence or absence of rDNA introns. These three types of differences provide an excellent basis for differentiating among individual strains of this genus. In a sample of 53 strains representing 12 lineages referred to as sequence types, the 18S rDNAs differed in 52 (98%) of the isolates (3, 7, 9).

We compared the 18S rDNA base sequences from three isolates, H30 (AcPHL/7a), H31 (AcPHL/7b), and H32, from the right eye of the patient studied by Kilvington et al. (6). The methods used for DNA isolation, PCR amplification, and 18S rDNA sequencing are described elsewhere (3, 9, 10). H30, a January isolate, was collected before therapy, and H31, a February isolate, was collected 4 weeks after therapy (6). The March isolate from the original investigation was missing; H32 (a July isolate) was used instead.

Drug sensitivity testing originally used a mixed trophozoite and cyst culture and a double-dilution technique in a tube (2). At that time, H30 was sensitive to propamidine at a MIC of 1.5 μg/ml and a minimum cysticidal concentration (MCC) of 12.5 μg/ml. The posttherapy isolate H31 and the March isolate were not cultivatable at 37°C, but grew at 25°C and were resistant to propamidine (MIC, 12.5 μg/ml; MCC, 50 μg/ml).

In the present study, which included H30, H31, and H32 (a July isolate), the sensitivities of trophozoites and cysts were tested separately by a microtiter method (5). Trophozoites and

* Corresponding author. Mailing address: Department of Molecular Genetics, The Ohio State University, 484 W. 12th Ave., Columbus, OH 43210-1292. Phone: (614) 292-5963. Fax: (614) 292-4466. E-mail: byers.2@osu.edu.

† Present address: Department of Genetics, University of Pennsylvania, Stellar Chance Labs, Philadelphia, PA 19104.

TABLE 1. Locations of sequence differences between the *RnsA*, *-B*, and *-C* alleles in strains H30, H31, and H32 and their relationship to the single *Rns* allele of ATCC 50497[a]

Allele	Sequence at nucleotide position (bp)											
	289	290	891	894	895	896	897	902	903	904	905	906
ATCC 50497	-[b]	-	-	T	G	-	-	C	-	-	-	-
RnsA	-	-	-	T	G	-	-	C	-	-	-	-
RnsB	A	C	-	T	G	-	-	C	-	-	-	-
RnsC	A	C	G	G	C	G	T	A	C	C	G	T

[a] Comparisons are for complete genes minus the terminal sequences used for PCR amplification. Nucleotide positions are for *RnsC*. The sequence of ATCC 50497 is found under GenBank accession no. U07410.

[b] -, gap created at this position by sequence alignments.

cysts of H30 both were sensitive to propamidine at a minimum trophozoite amoebicidal concentration (MTAC) of 3.2 μg/ml and an MCC of 12.5 μg/ml (5). H31 and H32 were resistant to propamidine at an MTAC of 25 μg/ml and MCCs of 50 and 100 μg/ml, respectively. Similar values were obtained for isolates retested after the molecular studies. All isolates grew at 25°C. H30 grew optimally at 37°C, but neither H31 nor H32 grew at 37°C.

Initial sequencing results indicated that more than one 18S rDNA sequence was present in each of the stock cultures of H30, H31, and H32. This was evidence for more than one strain in each culture or for more than one rDNA allele in each strain. To distinguish between these possibilities, single amoebae were cloned from the stock cultures. The 18S rDNA was obtained by PCR from the amoeba clones and then cloned in bacterial hosts. Sequencing of the cloned 18S rDNA revealed that three alleles, *RnsA*, *RnsB*, and *RnsC* (Table 1), were present in all amoebae in all clones from H30, H31, and H32. The observation that propamidine-sensitive and -resistant isolates had identical alleles is strong evidence that all isolates belong to the same strain.

Analysis of the three *Acanthamoeba* keratitis isolates examined here strongly supports the previous work of Kilvington et al. (6). Both laboratories concluded that propamidine-resistant amoebae obtained during drug therapy were from the same strain as amoebae obtained prior to therapy. Thus, it is probable that resistance was due to a genetic or physiological change that occurred during therapy. The possibility that resistance developed because of preferential multiplication of resistant amoebae from a second strain originally present in a mixed infection is very unlikely. There was no evidence for a second strain in any of the isolates. The existence of multiple alleles in the infectious strain was uncommon for *Acanthamoeba*, but cell cloning established clearly that all three alleles are present in all amoebae in each of the isolates. Thus, only one strain was isolated from the infection. The data cannot rule out the possibility that a drug-resistant mutant amoeba or subpopulation was present at the time of infection. If this was so, however, it is clear that a significant increase in propamidine resistance was a response to therapy.

We thank John Hay for maintaining the cultures at the Tennent Institute, for retesting the propamidine sensitivities of the amoeba strains used, and for many helpful discussions.

The work of D.R.L. and T.J.B. was supported by Public Health Service grant EY09073 from the National Eye Institute.

REFERENCES

1. **D'Aversa, G., G. A. Stern, and W. T. Driebe.** 1995. Diagnosis and successful medical treatment of *Acanthamoeba* keratitis. Arch. Ophthalmol. **113:**1120–1123.
2. **Ficker, L., D. Seal, D. Warhurst, and P. Wright.** 1990. *Acanthamoeba* keratitis: resistance to medical therapy. Eye **4:**835–838.
3. **Gast, R. J., D. R. Ledee, P. A. Fuerst, and T. J. Byers.** 1996. Subgenus systematics of *Acanthamoeba*: four nuclear 18S rDNA sequence types. J. Eukaryot. Microbiol. **43:**498–504.
4. **Gautom, R. K., S. Lory, S. Seyedirashti, D. L. Bergeron, and T. R. Fritsche.** 1994. Mitochondrial DNA fingerprinting of *Acanthamoeba* spp. isolated from clinical and environmental sources. J. Clin. Microbiol. **32:**1070–1073.
5. **Hay, J., C. M. Kirkness, D. V. Seal, and P. Wright.** 1994. Drug resistance and *Acanthamoeba* keratitis: the quest for alternative anti-protozoal chemotherapy. Eye **8:**555–556.
6. **Kilvington, S., J. R. Beeching, and D. G. White.** 1991. Differentiation of *Acanthamoeba* strains from infected corneas and the environment by using restriction endonuclease digestion of whole-cell DNA. J. Clin. Microbiol. **29:**310–314.
7. **Ledee, D. R., J. Hay, T. J. Byers, D. V. Seal, and C. M. Kirkness.** 1996. Molecular characterization of *Acanthamoeba griffini*: a new corneal pathogen. Invest. Ophthalmol. Vis. Sci. **37:**544–550.
8. **Seal, D. V., J. Hay, C. M. Kirkness, A. Morrell, A. Booth, A. Tullo, A. Ridgeway, and M. Armstrong.** 1996. Successful medical therapy of *Acanthamoeba* keratitis with topical chlorhexidine and propamidine. Eye **10:**413–421.
9. **Stothard, D. R., J. M. Schroeder-Diedrich, M. H. Awaad, R. J. Gast, D. R. Ledee, S. Rodriguez-Zaragoza, C. L. Dean, P. A. Fuerst, and T. J. Byers.** 1998. The evolutionary history of the genus *Acanthamoeba* and the identification of eight new 18S rRNA gene sequence types. J. Eukaryot. Microbiol. **45:**45–54.
10. **Weekers, P. H. H., R. J. Gast, P. A. Fuerst, and T. J. Byers.** 1994. Sequence variations in small subunit ribosomal RNAs of *Hartmannella vermiformis* and their phylogenetic implications. Mol. Biol. Evol. **11:**11684–11690.
11. **Wright, P., D. Warhurst, and B. R. Jones.** 1985. *Acanthamoeba* keratitis successfully treated medically. Br. J. Ophthalmol. **69:**778–782.
12. **Yagita, K., and T. Endo.** 1990. Restriction enzyme analysis of mitochondrial DNA of *Acanthamoeba* strains in Japan. J. Protozool. **37:**570–575.