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In vitro studies on susceptibility of *Acanthamoeba castellanii* to selected chemical agents

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Abstract

Amoebae of the *Acanthamoeba castellanii* Neff strain, cultured in bacteria-free condition at room temperature, were tested *in vitro* for their susceptibility to selected chemical compounds. The amoebae, grown during 4 and 10 days following regular subculturing, were exposed for 24 h to three compounds: the newly synthesized imidazole derivatives (1) 2-methyl-3N-(4-nitrobenzyl)-5-nitro-1H-imidazole designated as AG16; (2) 2-methyl-3N-(3,5-dinitrobenzyl)-5-nitro-1H-imidazole designated as AG17 and to the (3) cationic antiseptic agent chlorhexidine digluconate (CHX). Two different concentrations of the above-mentioned substances were applied. The results showed that all chemicals tested had some amoebicidal effect on 4-day grown population of *Acanthamoeba*; variations in susceptibility of the amoebae, depending on the kind and concentration of the compounds occurred. It was also observed that several agents changed the relationship between *A. castellanii* trophozoites and cysts. Chlorhexidine digluconate caused a decrease in percentage of the trophozoites to 88.4% at higher concentration of the compound, 10 μg/ml (in comparison to 97.8% from the control samples), however, significant increase of the cyst percentage, to 11.6% in assays with this concentration of chlorhexidine were revealed (2.2% in the control samples). Tendency toward induction of encystment was also apparent when AG17 was applied. Because activation of the dormant cysts can lead to repeated development of amoebae, very important is cysticidal efficacy of tested agents. Among all compounds examined by us, the newly synthesized imidazole derivative AG16 was the most effective. It was expressed as decrease in average number of amoebae (to 655.6×10^3/ml at concentration of the agent 8 μg/ml; 682.5×10^3/ml in assays with CHX at concentration 4 μg/ml; 893×10^3/ml in the control samples) connected with a clear cysticidal effect (1.4% of cysts at concentration 4 μg/ml of AG16).

Key words

Acanthamoeba castellanii, Protozoa, susceptibility to chemicals, ultrastructure

Introduction

Different strains of amoebae belonging to the genus *Acanthamoeba* are known from many parts of the world as free-living organisms. The amoebae have been demonstrated in soil and air as well as in fresh, sea, chlorinated, mineral and tap water; additionally, they have been isolated from dental irrigation systems, hospital environment, air conditioning units, ventilation as well as vegetables and various animal organisms (Larkin *et al*. 1990, Walochnik *et al*. 1999, Khan 2003, Schuster and Visvesvara 2004a). However, the trophozoites and cysts of the amoebae may also exist as parasites. This is why the primary free-living amoebae are considered to be amphizoic organisms. They may be causative agents of human diseases affecting skin, eyes, lungs and paranasal sinuses. There is particularly serious risk to human health and life, when pathogenic strains of *Acanthamoeba* species infect human brain causing granulomatous, amoebic encephalitis (GAE). Moreover, different species of *Acanthamoeba* may serve as vectors for bacteria, fungi and protozoa, such as some species of *Legionella*, *Escherichia*, *Chlamydia*, *Pseudomonas, Candida* or *Cryptosporidium*; thus they play an important

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role in the environmental transmission and dispersion of potentially pathogenic endosymbiotic microorganisms (Essig *et al*. 1997; Walochnik *et al*. 1999, 2002; Winiecka-Krusnell and Linder 2001; Ares-Mazas *et al*. 2004; Bartolome *et al*. 2004; Schuster and Visvesvara 2004a, b). The infections caused by *Acanthamoeba* species may occur in both immunocompetent and immunocompromised individuals, but they are reported in the latter predominantly. Infections also occur in the persons with systemic diseases undergoing immunosuppressive therapy and in patients with HIV/AIDS (Visvesvara 1993, Martinez and Visvesvara 1997, Casper *et al*. 1999, Van Hamme *et al*. 2001, Schwarzwald *et al*. 2003). For this reason, the amoebae have been recognized as opportunistic microorganisms (Martinez 1980, Teknos *et al*. 2000, Schuster 2002, Schuster and Visvesvara 2004a, b). We also demonstrated trophozoites and cysts of *Acanthamoeba* species accompanying infections with *Entamoeba gingivalis* in the oral cavity of 4 among about 100 patients examined with or without systemic diseases. The patients also showed deterioration of the periodontium and gingiva (Chomicz *et al*. 2000, 2001, 2002).

Despite advances in chemotherapy, acanthamoebic keratitis, a non-opportunistic serious eye disease, is reported with increasing frequency in various parts of the world, particularly in contact lens wearers (Rodriguez-Zaragoza and Magana-Becerra 1997; Walochnik *et al*. 2000a; Garcia 2001; Kilic *et al*. 2004; Schuster and Visvesvara 2004a, b). Diagnosis is by microscopic visualization of amoebae in stained slides prepared directly from corneal scraping or by cultivation of the amoebae from samples. Treatment of the diseases caused by *Acanthamoeba* is difficult and results of therapy applied are often disappointing.

Treatment with chlorhexidine is usually used in treating *Acanthamoeba* keratitis, but relapse with culture-positive isolation of *Acanthamoeba* may occur. Combination drug therapy with the antimicrobial agents chlorhexidine gluconate and polyhexamethylene biguanide and other antimicrobials (e.g., hexamidine) has been used more or less successfully (Kilvington *et al*. 2002, Pérez-Santonja *et al*. 2003). Drug treatment was effective for isolates of *Acanthamoeba* from southern Africa and the UK (Niszl and Markus 2001), but cases with *in vivo* resistance to polyhexamethylene biguanide were reported from New Zealand (Murdoch *et al*. 1998). Clinical isolates of *A. polyphaga* were susceptible to some membrane-active peptide compounds. Clotrimazole and ketoconazole had cysticidal activity *in vitro* at high concentrations of the agents. Recent studies have shown that the trophozoites and cysts of clinical isolates of *Acanthamoeba* differ in their pathogenicity and susceptibility to various drugs, such as chlorhexidine, diminazine, clotrimazole, polyhexamethylene biguanide, neomycin and, especially, to combinations of some drugs (Ferrante 1991, Schuster and Jacob 1992, Schuster 1993, Garcia 2001, Lloyd *et al*. 2001, Walochnik and Aspöck 2001, Khan 2003, Schuster and Visvesvara 2004a, b).

Due to the toxicity of high concentrations of the compounds tested, development of drug resistance and contradictory results from drug testing, an optimal strategy for antiacanthamoebic treatment is not yet defined and further studies on susceptibility of *Acanthamoeba* to different chemical agents are needed.

In our previous study (Kopańska *et al*. 2004), we screened benzimidazole and benzotriazole derivatives for their *in vitro* antiamoebic activity. The aim of this study was to test *in vitro* effects of different concentrations of the antiseptic agent chlorhexidine and selected chemical compounds on the dynamics and viability of developmental stages of the Neff strain of *Acanthamoeba castellanii*.

Materials and methods

Amoebae of the *A. castellanii* (Neff strain), grown at 22–26°C in bacteria-free cultures in sterile 15-ml tubes containing BSC culture medium (Černa and Novak 1968) enriched with 10% calf serum, were used in the studies. The amoebae were subcultured twice a month. Amoebae used in all assays were grown for 4 and 10 days following regular subculturing.

The axenically grown amoebae were exposed to the three compounds: the newly synthesized imidazole derivatives: (1) 2-methyl-3N-(4-nitrobenzyl)-5-nitro-1H-imidazole designated as AG16; (2) 2-methyl-3N-(3,5-dinitrobenzyl)-5-nitro-1H-imidazole designated as AG17; and to (3) the cationic antiseptic agent chlorhexidine digluconate. Two different concentrations of the above-mentioned substances were applied and their effect examined: 4 µg/ml and 8 µg/ml both of AG16 and AG17, and 4 µg/ml and 10 µg/ml of chlorhexidine digluconate; concentrations of the latter agent were similar to those that have been applied in our previous studies (Kopańska *et al*. 2004). For both the compound susceptibility and the control assays, 1 ml of the vortexed culture containing *A. castellanii* was transferred to individual 1.5-ml Eppendorf tubes. The assays were performed at 23°C. A 10 µl dilution of the new compounds in dimethyl sulfoxide (DMSO) or DMSO without agent were added to part of those tubes; the same quantity of chlorhexidine digluconate was used in assays with the antiseptic agent. It has been determined that 10 µl DMSO added to 1 ml of culture medium containing *Acanthamoeba* has an insignificant effect on number and status of the amoebae. Thus, such a concentration of DMSO was used in proper assays. After 24 h of exposure to the above-mentioned three agents, the tested cultures were intensively vortexed, and then 20-µl samples were taken from each of them for preparation of wet slides.

The status of the surviving *Acanthamoeba* was assessed microscopically and compared with that observed in the control cultures. Wet-mount slides were prepared in like manner as it was in our previous studies; cover slips of 24 × 24 mm and 10 × 10 magnification were applied to count the trophozoites and cysts. Choice of this way of examination, without use of hemocytometer or other counting chamber, being in agreement with recommendations of diagnostic parasitology and with our own experiences; this mode of examination allows to compare the results with clinical material. Percentage of particular stages of the *Acanthamoeba* was assessed. Mean values of six counts calculated for 1 ml of culture medium were counted and compared for each concentration of the tested compounds as well as the control assays.

Because the mode of action of therapeutic agents against *Acanthamoeba* is not exactly known, electron microscope examinations were also undertaken to determine the effect of the compounds used at the ultrastructural level. Samples of the tested cultures were centrifuged and the sediment was fixed in a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 2 h at room temperature. The material was postfixed in 1% osmium tetroxide for 2 h, dehydrated in a graded ethanol series, infiltrated with propylene oxide and embedded in Spurr's epoxy resin. Ultrathin sections, mounted on formvar-coated specimen disks, double stained with lead citrate and uranyl acetate, were examined with a transmission electron microscope (JEM 1200EX).

Results

Assessment of developmental dynamics of the control cultures showed that the amoeba population was in log phase growth at 4 and 10 days following regular subculturing. General numbers of amoebae calculated for 1 ml of the control culture medium reached an average of 893 × 10^3 in the 4-day cultures and 1707 × 10^3 in those grown 10 days. In general, about 1600 ×10^3 of amoebae were directly included in the experiments and more than 50 assays realized. Microscopic examinations of the wet-mount slides prepared for assessment of amoebae both of the control cultures of *Acanthamoeba* grown in bacteria-free condition and those that were exposed for 24 h to chemical agents revealed some differences between the untreated and treated amoebae. All chemicals used *in vitro* had some amoebicidal effect on 4-day grown population of the protozoans. It was expressed as appearance of the dividing amoebae only sporadically and as distinct decrease in general number of the protozoa.

Comparative assessment of the results showed also that several agents changed the relationship between *A. castellanii* trophozoites and cysts. It was shown that various compounds affected particular amoeba stages in different ways.

Chlorhexidine digluconate caused a decrease in percentage of the trophozoites. The amoebicidal effect was more intense at the higher concentration (10 µg/ml) of the antiseptic agent: 88.4% trophozoites in comparison to 97.8% from the control samples. The newly synthesized imidazole derivative AG16 was the most effective *in vitro* among all agents examined by us. The reduction of viability of the *A. castellanii* population was expressed as a clear decrease in general average number of amoebae to 655.6 × 10^3/ml in assays with AG16 (682.5 × 10^3/ml with chlorhexidine, 893 ×10^3/ml in the control assays). Comparison of the percentage of particular stages of *A. castellanii* indicated that in spite of the general amoebicidal effects, encystment was induced by some of the agents used. The most significant increase of the cyst level was caused by chlorhexidine digluconate; at higher concentration of the agent, 10 µg/ml, the percentage of cysts was more than 5-times higher in comparison with those of control assays. It is likely that maintenance of relatively high average number

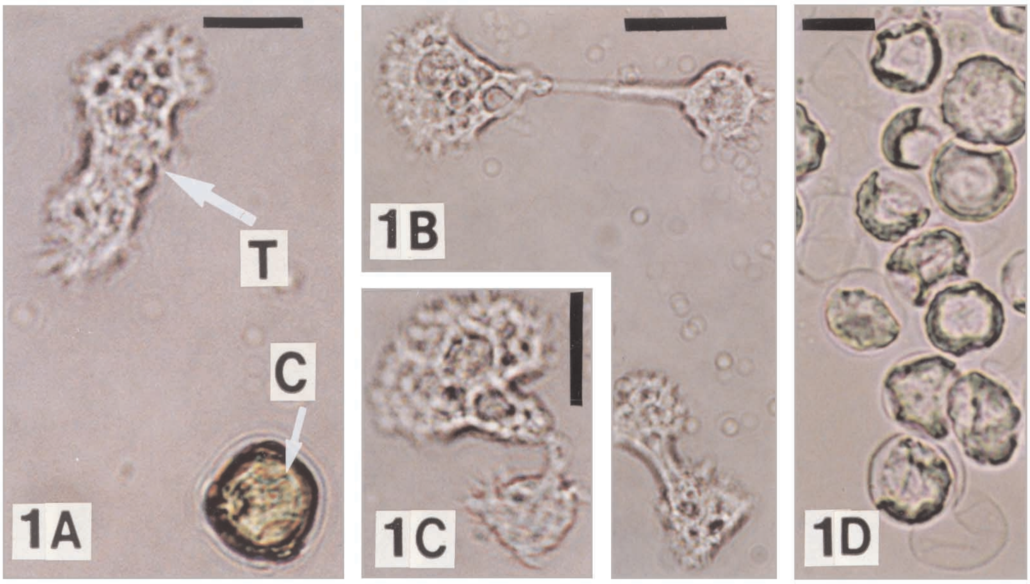
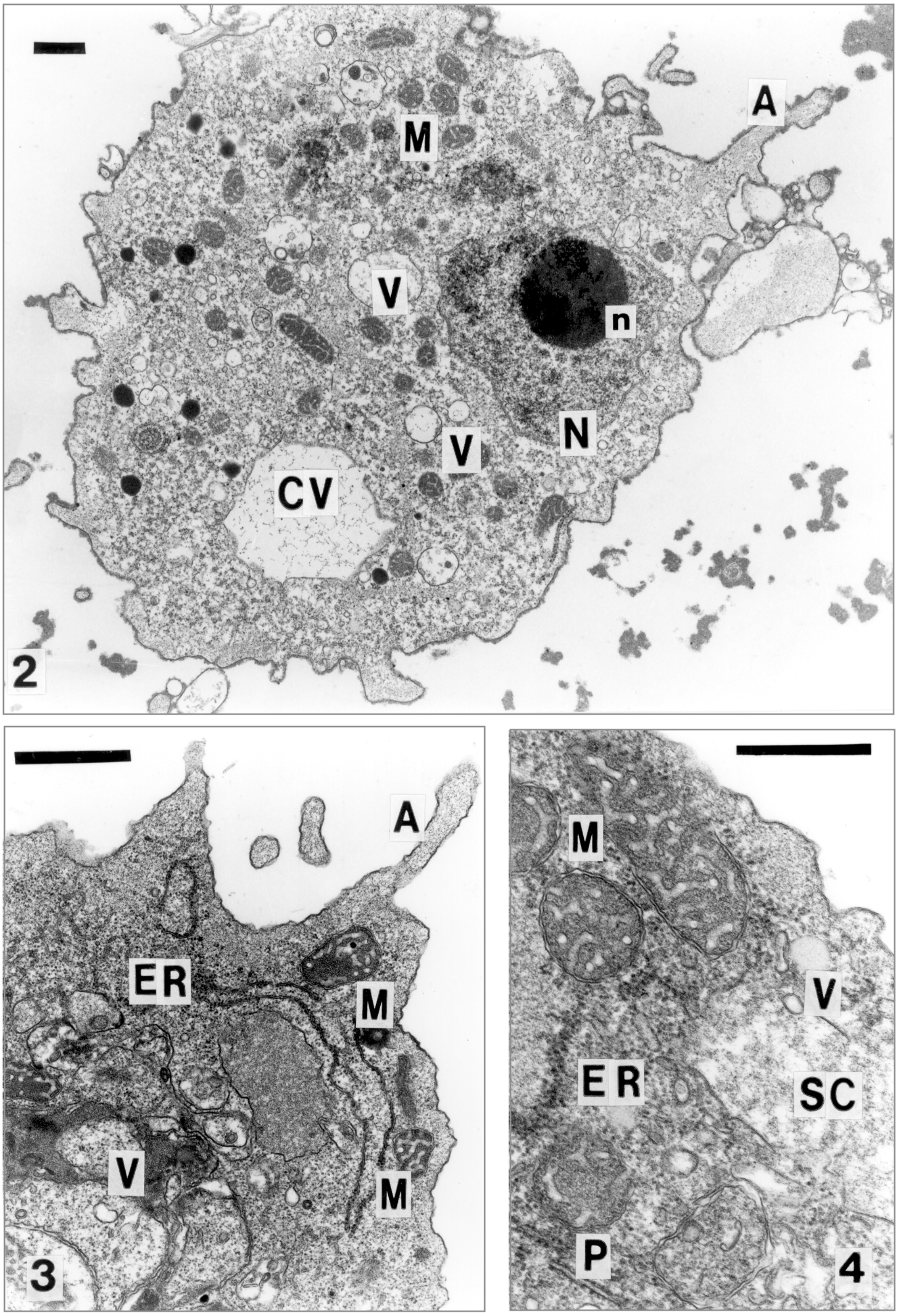


Fig. 1A-D. Light micrographs showing amoebae found in wet-mount slide preparations. **A.** *Acanthamoeba castellanii* from the control sample. Note characteristic acanthopodia at functionally posterior part of the trophozoite (T) and the double-walled cyst (C) with pores. **B.** Dividing *A. castellanii* trophozoite in10-day grown sample with addition of AG17. **C.** The same trophozoite at the end phase of its division. **D.** Cysts of *A. castellanii* in sample with AG16; note some deformed, crescent-shaped or broken cysts. Scale bars = 10 µm



Figs 2–4. Transmission electron micrographs showing *Acanthamoeba castellanii* from the control and tested samples. **2.** Cross-section through an amoeba from the control sample. Note acanthopodia (A), nucleus (N) with nucleolus (n), contractile vacuole (CV), vacuoles (V), mitochondria (M). **3.** Cross-section through the fragment of protozoan cytoplasm from the assay with AG16. Note extensive endoplasmic reticulum (ER), numerous, various in shape and size, moderate electron-dense vacuoles (V). **4.** Fragment of amoeba cytoplasm from the assay with chlorhexidine; note abundance of mitochondria, polyribosomes (P), sol-like cytoplasm (SC). Scale bars = 1 µm

Table I. Comparison of the content of trophozoites and cysts *A. castellanii* in the cultures grown for 4 days after 24 h incubation with different chemical compounds

Compounds	Concentrations (μg/ml)	Number of amoebae*	% of particular stages trophozoites	cysts
CHX in H_2O	4.0	682500 ± 75.0	95.3	4.7
	10.0	780700 ± 72.2	88.4	11.6
AG16 in DMSO	4.0	767000 ± 62.0	98.55	1.45
	8.0	655600 ± 59.2	97.5	2.5
AG17 in DMSO	4.0	721000 ± 70.4	95.0	5.0
	8.0	850000 ± 72.4	97.3	2.7
C**	–	893000 ± 76.7	97.8	2.2

CHX – chlorhexidine digluconate, AG16 – 2-methyl-3N-(4-nitrobenzyl)-5-nitro-1H-imidazole, AG17 – 2-methyl-3N-(3,5-dinitrobenzyl)-5-nitro-1H-imidazole, DMSO – dimethyl sulfoxide. *Values are made of six counts, calculated for 1 ml of culture medium ± SD. **The value is mean for control culture, culture with addition of DMSO or H_2O.

of amoebae was associated with clear induction of encystment appearing at this concentration of chlorhexidine. Tendency toward induction of encystment was also apparent when a low concentration of the new imidazole derivative AG17 was used.

Contrary to that described above, a decrease in general number of amoebae, caused by the new imidazole derivative AG16, to 767 × 10^3/ml in comparison with 893 × 10^3/ml in the control assays, was associated with a cysticidal effect. It has been demonstrated in the samples with the low concentration of the compound, 4 μg/ml, that the percentage of *A. castellanii* cysts was decreased to 1.45% (2.2% in the control assays) and lower than this from assays with chlorhexidine (4.7% at concentration 4 μg/ml and 11.6% at 10 μg/ml). A comparison of the content of trophozoites and cysts *A. castellanii* in the cultures grown for 4 days after 24 h incubation with different chemical compounds is presented in Table I.

Simultaneously to differences in the relationships between trophozoites and cysts of *A. castellanii*, significant changes in appearance and movement of the amoebae were observed in the assays with various compounds; they were visible already at low levels of the tested agents. Some of the living trophozoites moved more slowly and rounded up with or without sporadically formed acanthopodia. Clear changes caused by the chemical agents were also visible in the cyst stage. Typical double-walled cysts found in the control cultures consisted of outer ectocyst and inner endocyst; under the influence of the chemical agents some of the cysts became deformed, crescent-shaped or damaged. Cytoplasm remnants were visible inside the damaged cysts. Light micrographs showing *A. castellanii* found in wet-mount slides are presented in Figure 1.

Transmission electron microscope (TEM) examinations revealed also morphological changes at the ultrastructural level in some trophozoites taken from the samples with compounds tested (Figs 2–4). It was found that homogeneous or sol-like cytoplasm was visible only sporadically and in those scattered places in which the acanthopodia appeared. The moderate dense, gel-like cytoplasm was limited with a plasma membrane that created relatively thick, rounded contour of the trophozoite. After 24 h exposure to the agents tested, the gel-like *Acanthamoeba* cytoplasm of the trophozoites showed presence of extensive rough endoplasmic reticulum, numerous free ribosomes and polysomes, well-developed Golgi complex, and a nucleus with prominent nucleolus and heterochromatin islands. There were also agglomerations of a significant number of mitochondria with tubular cristae, abundance of vacuoles filled with amorphous material, as well as numerous small vesicles. The cytoplasmic features were less expressed in trophozoites of the control samples: cell organelles, particularly vacuoles were not so numerous.

It was striking that the amoeba population grown 10 days following regular subculturing showed weak reaction to the chemicals tested. Generally, an induction of amoeba divisions was observed resulting in a more or less, but usually no more than several percent increase of the general number of the amoebae. Simultaneously, there was weak encystment tendency; no reduction of viability of *A. castellanii* population was observed in the 10-day grown assays in comparison with those in control cultures grown through the same time interval.

Discussion

Many chemical agents were tested, also *in vitro*, for the drug susceptibility of different species, strains and isolates of *Acanthamoeba* (Schuster and Jakob 1992; Gatti *et al.* 1998; Turner *et al.* 2000; Walochnik *et al.* 2000a, b, 2002, 2004; Garcia 2001; Aksozek *et al.* 2002; Hughes *et al.* 2003; Pérez-Santonja *et al.* 2003; Schuster *et al.* 2003; Seal 2003a, b; Kopańska *et al.* 2004; Schuster and Visvesvara 2004a, b). The mode of action of therapeutic agents against *Acanthamoeba* species is poorly known. On the basis of studies using animal models (e.g., mice) it has been reported that some changes in architecture of the cell membrane leading to its increased permeability can be associated with sterol metabolism (Schuster and Visvesvara 2004b). Many factors, such as virulence of amoeba population, phase of infection, conditions of culturing or immune status of infected host, as well as kind and concentration of the chemicals tested, are known to determine variability in effects of drugs on trophozoites and cysts of various strains even of the same species of *Acanthamoeba*. In addition, contradictory results have been reported by various researchers and different views are often presented. Generally, there are no established standards for testing the susceptibility of amoebae to chemical agents (Schuster and Visvesvara 2004b).

Results of our *in vitro* studies revealed that several stages of *A. castellanii* showed variations in susceptibility, depending on the kind and concentration of the compounds tested as well as developmental phases of the amoeba population.

All agents examined indicated some amoebicidal effect on a population of the *A. castellanii* grown for 4 days: dividing amoebae were found not so often, many trophozoites became rounded, without acanthopodia and a clear decrease in general number of the amoebae appeared. However, it was revealed by TEM that the ultrastructure of the affected trophozoites reflected a high level of metabolism that was expressed in extensive growth of rough endoplasmic reticulum, numerous polysomes and free ribosomes and abundance of mitochondria with tubular cristae, well-developed Golgi complexes, numerous vacuoles and small vesicles.

Simultaneously, in spite of the fact that some cysts showed clear deformations at relatively low levels of the tested agents, it was found that a significant increase of the cyst level was induced by chlorhexidine digluconate, and a tendency to encystment induction was also caused by the new imidazole derivative AG17.

Recently, it is emphasized as particularly important in the *in vitro* studies on the susceptibility of free-living opportunistic amoebae to chemicals to distinguish between amoebostatic and amoebicidal effects. It was reported that some agents and drugs used can induce encystment that subsequently, by excystment, lead to repeated development of amoebae. It is in agreement with known facts that the double-walled cysts of *Acanthamoeba* are highly resistant to antimicrobial and antiparasitic drugs as well as environmental factors: they can maintain their viability and virulence for as long as 25 years. It has been emphasized that activation of the dormant cysts can lead to recurrence of infection; thus, apart from the amoebicidal effect, cysticidal efficacy of the tested compounds should be assessed (Kilvington *et al*. 1990, Mazur *et al*. 1995, Garcia 2001, Aksozek *et al*. 2002, Khan 2003, Schuster and Visvesvara 2004b).

The results obtained from our studies revealed clear decrease of percentage trophozoites induced by chlorhexidine that was more intense at higher concentration of the chemical agent (10 µg/ml). However, this commonly used antiseptic agent induced encystment reaction, thus, repeated development of populations of *A. castellanii* cannot be excluded.

It is noteworthy that among the new imidazole derivatives, the most effective in reducing the number of surviving amoebae was AG16. The amoebicidal activity of the compound was connected with a clear cysticidal effect. The highest reduction in number of cysts was when a lower concentration of AG16, 4 µg/ml was used. Among all substances tested in these *in vitro* studies, the new imidazole derivative AG16 seems very promising not only due to its amoebicidal activity, but also because of clear cysticidal effects.

This is the first report presenting results of our *in vitro* study on *A. castellanii* with use the two newly synthesized imidazole derivatives. For this reason, only selected aspects of complicated antiamoebic effects on dynamics and viability of the amoebae, depend on the kind and concentration of used agents as well as phase of population development, were assessed and compared with these of the cationic chlorhexidine digluconate and the controls. The results presented suggest that further studies in this field will be very helpful for assessment of differentiation of the surviving amoeba population, explanation of the mechanisms of the amoebicidal activity of the tested compounds, as well as of the significance of various environmental factors for resistance or susceptibility of *A. castellanii* to several chemical agents.

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