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In-vitro Effects of Benzalkonium-ion Intercalated Aluminium Triphosphate on the Second-stage Larvae and Fertilized Eggs of *Toxocara canis*

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Abstract

We evaluated the effectiveness of benzalkonium-ion intercalated aluminium triphosphate (BIAT) *in vitro* as a larvicide against the second-stage larvae and fertilized eggs of *Toxocara canis* (*T. canis*). More than 6.25 mg/ml of BIAT killed second-stage *T. canis* larvae within 30 minutes, and all larvae observed died at the concentration of 0.025 mg/ml after 6 hours of incubation with BIAT. In addition, BIAT had a larvicidal effect against larvae in eggs when they were incubated with BIAT from the beginning of their development. However, this effect was not observed with eggs that had already matured into infective larvae. These data suggest that chemical sterilization of *T. canis* might be achieved with use of BIAT.

Key word: *Toxocara canis*, benzalkonium, environmental hazard, sandpits, larvicidal compound

Introduction

The results of epidemiological surveys have drawn attention to the importance of sandpits contaminated with the eggs of the *Toxocara* species as an infectious source of toxocariasis. Many investigators reported that 13 to 92% of sandpits in parks were contaminated by the eggs (Dada and Lindquist, 1979; Dunsmore *et al.*, 1084; Düwel, 1984; Horn *et al.*, 1990; Jansen *et al.*, 1993; Shimizu, 1993; Uga *et al.*, 1989; Uga, 1993; Valkounova, 1982a, 1982b). Moreover, some sandpits contained infective larvae which were the causative agent of visceral larva migrans. Although a few conventional control measures, such as steam sterilization (van Knapen, *et al.*, 1979) or permanent fencing around the sandpit (Düwel, 1984) have been successful, treatment either needs to be repeated or is costly.

In this paper, we report a new approach to sterilizing sand contaminated by *T. canis* eggs. The aim of the present study is to reduce the risk of *T. canis* infection from sandpits in public parks and playgrounds.

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Materials and Methods

T. canis

We obtained adult female worms of *T. canis* from infected dogs. Fertilized eggs were collected from the uterus of the worms. The eggs were then kept in 0.5% formaldehyde solution at 30°C for 4 weeks. They were then washed free of formaldehyde, treated with sodium hypochlorite, and hatched as second-stage larvae by the method described by Kondo *et al.* (1981). The larvae were collected and maintained in a culture medium, Dulbecco's Modification of Eagle's Medium, (Flow Laboratories, Irvine, Scotland) at pH 7.2 at 37°C with weekly replacement of the medium. Some fresh, fertilized eggs were used in the growth inhibition test.

Chemical

Benzalkonium-ion intercalated aluminium triphosphate (BIAT) was kindly supplied by Rasa Kogyo Co. Ltd. (Tokyo, Japan). The supplier reports the synthesis process to be as follows. BIAT was synthesized by suspending 10 g of aluminium

triphosphate in 500 ml of 1% benzalkonium chloride solution with 0.5% n-butylamine. The suspension was stirred for 2 hours at room temperature to form the intercalation compound. The resultant product was filtered, washed with distilled water, and dried. The compound was a white slightly water-soluble powder.

Assay

For assays of larvae killed by BIAT, the larvae were incubated with serial dilution of 25 mg/ml BIAT in 24-well microplates (Corning, USA). The BIAT was dissolved in 2% W/W dimethyl sulfoxide (Sigma Chemical Company, USA), and diluted in 0.1 M phosphate buffered saline solution (PBS, pH 7.2) to keep the dimethyl sulfoxide concentration at 2%. The larvae were then incubated with serial diluted BIAT at 30°C for the designated periods. After incubation, some larvae in each well was plated in 1.5 ml-polypropylene microcentrifuge tubes containing 1 ml of PBS. The larvae were then centrifuged, and supernatant fractions were aspirated and discarded. The larvae were suspended in PBS again and put on glass slide and covered with glass measuring 22×22 mm. The slides were subsequently incubated at 37°C for 10 min. After this incubation, the activity of the larvae was assessed and expressed by the mobility index described previously (Kiuchi *et al.*, 1987).

To evaluate the effect of BIAT on *T. canis* eggs during development, the eggs incubated with BIAT at 30°C for 2 weeks, and then washed free from BIAT with PBS. The viability of the larvae in the eggs was assessed by gently crushing the eggshell and determining their mobility indices (Kiuchi *et al.*, 1987). Embryonated eggs were obtained by incubating fertilized eggs for 3 weeks at 30°C and the effects of BIAT on these embryonated eggs was examined. The embryonated eggs were incubated with BIAT at 30°C for 30 days, and then the mobility indexes of the infective larvae were calculated as described previously (Kiuchi *et al.*, 1987).

Results

Larvicidal effects of BIAT

In this assay, we attempted to assess the effect of BIAT on the larvae at concentrations ranging from 25 mg/ml to 0.025 mg/ml. However, we were unable to observe the larvae in the wells that contained more than 12.5 mg/ml of BIAT. Because the residual BIAT in the microcentrifuge tube affected larval mobility indices and the supernatant was still cloudy even after washing three times with PBS at these concentrations, the data for 12.5 mg/ml or more were not shown.

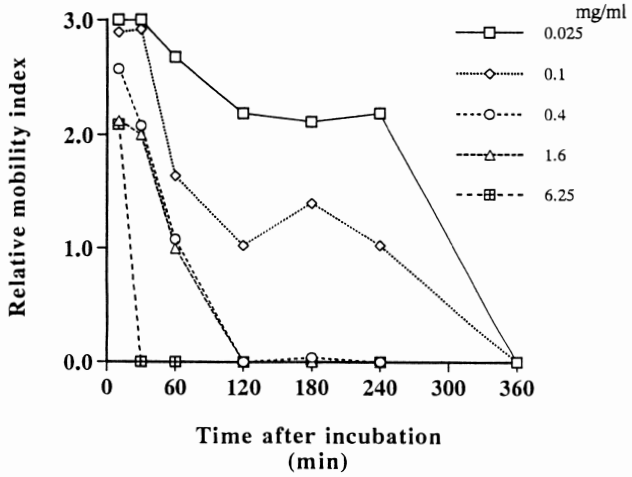
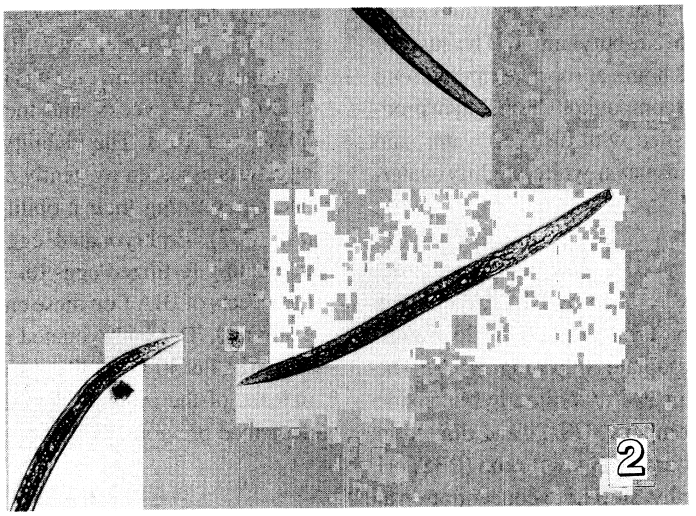


Fig. 1 Dose dependent larvicidal effect of benzalkonium-ion intercalated aluminium triphosphate on second-stage larvae of *Toxocara canis*.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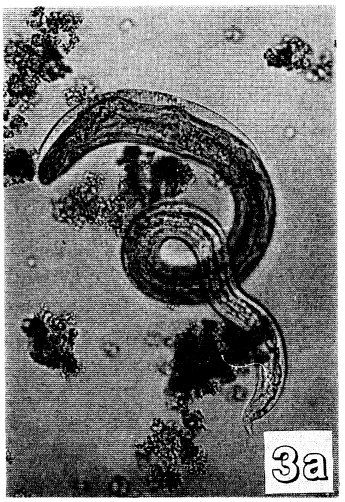
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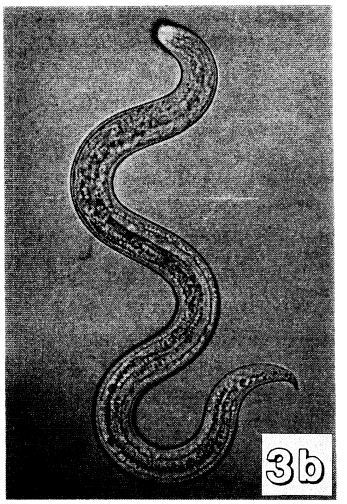
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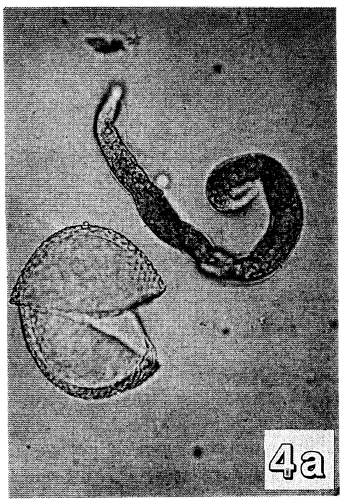
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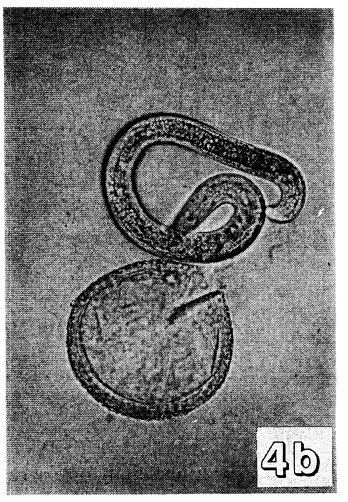
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Figure 1 shows the dose-dependent larvicidal effect of BIAT *in vitro*. At a concentration of 6.25 mg/ml, BIAT killed all larvae within 30 min. We noticed the larvae quavered intensely immediately after contact with BIAT, then stopped their activity. Regardless of the concentration of BIAT, all larvae died in a straight form after 6 hours of incubation (Fig. 2). However, some larvae appeared to swell at a concentration of 3.2 mg/ml or more before they died (Fig. 3).

Effects of BIAT on T. canis *eggs*

We evaluated the ability of BIAT to inhibit the growth of fresh eggs of *T. canis*. Fertilized *T. canis* eggs became embryonated eggs containing larvae 2 weeks after incubation with BIAT at a concentration of 3.2 mg/ml or more at 30°C. However, the larvae in the eggs were obviously degenerative, and were already dead when artificially hatched (Fig. 4). On the other hand, BIAT at concentrations up to 6.25 mg/ml had no effect on larvae in eggs that had already become embryonated despite continuous incubation at 30°C for more than 30 days.

Discussion

Benzalkonium ion, the active ingredient of BIAT, is an anti-septic agent. The mode of action is assumed to be a long alkylate chain which destroys the bacterial cell membrane. However, benzalkonium ion is ordinarily used in solution, and, therefore, its persistence is limited. On the other hand, intercalated benzalkonium ion is thermostable and is not easily dissolved in water. These characteristics offer the advantage that BIAT dose not need to be applied more than once to the area requiring disinfection.

Benzalkonium ion also has a strong larvicidal effect on second-stage *T. canis* larvae (Prof. Tsuda, Y., Faculty of Pharmaceutical, Kanazawa University, personal communication). In the present study, we observed that the cuticle of some larvae became swollen when they came in contact with BIAT, suggesting the larvae, like bacteria, were directly impaired by the benzalkonium ion. However, almost all the larvae died in straight form without any morphological change after incubation with BIAT. This finding indicates that another mechanism might be involved in the larvicidal effect of BIAT.

BIAT killed the infective larvae in eggs when fertilized eggs were continuously incubated with BIAT starting at the beginning of their development, but was ineffective for larvae which had already matured. These findings suggest that BIAT might be effective only during the early stages of the developing egg. Further study is needed to clarify this point.

BIAT has already been used as an antiseptic agent for apparel and tableware. The administration of BIAT more than 20 g/kg of body weight dose not result in a survival period of mice, and no abnormality has been reported in the mutagenicity test and skin patch test, indicating the low toxicity of BIAT. These findings are important for use in the field.

BIAT has not only a strong larvicidal effect on second-stage *T. canis* larvae *in vitro*, but also on bacteria, especially *Staphylococcus aureus, Escherichia coli, Klebsiella pneuminiae* and *Pseudomonas aeruginosa* (Isquith, *et al.*, 1972) all of which may also be found in sandpits.

In a recent review of toxocariasis, Glickmann and Magnaval (1993) stated that there is no practical method available to purge the soil of *Toxocara* eggs. We believe, however, that chemical sterilization of sand is better than steam sterilization or fencing around sandpits in reducing the risk of *Toxocara* infection. In future studies we will attempt to ascertain the effect of BIAT on *Toxocara* eggs in a sandpit where fecal contamination has been established.

Fig. 2 All second-stage *Toxocara canis* larvae died within 6 hrs when they were incubated with benzalkonium-ion intercalated aluminium triphosphate (BIAT) at the concentration of 0.025 mg/ml.

Fig. 3 Some of the second-stage *Toxocara canis* larvae appeared to be swelling 30 minutes after incubation with benzalkonium-ion intercalated aluminium triphosphate (3.2 mg/ml, a). Larvae without BIAT showed normal appearance after the same incubation period (b).

Fig. 4 Degenerative dead larva in *Toxocara canis* egg after incubation with benzalkonium-ion intercalated aluminium triphosphate (3.2 mg/ml) at 30°C for 2 weeks (a). Fertilized eggs developed into normal embryonated eggs after the same incubation period (b).

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