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Effects of Milbemycin D on the Motility of *Angiostrongylus cantonensis* and *A. costaricensis**

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Introduction

Milbemycin D, one of 13 chemically-related antibiotics, was recently isolated from submerged cultures of *Streptomyces hygroscopicus* subsp. *aureolacrimosus* (Takiguchi *et al.*, 1980, 1983). The milbemycins were identified as a series of macrocyclic lactone derivatives (Takiguchi *et al.*, 1980) which are closely related to avermectins (Campbell, 1985). From the conspicuous resemblance in their chemical structures, it seems likely that these new antibiotics as well as avermectins have insecticidal, acaricidal and nematocidal properties. Indeed, insecticidal and acaricidal activities were detected in all milbemycins (Takiguchi *et al.*, 1980). Additionally, there have been some reports describing anthelmintic effects of milbemycin D on intestinal nematodes like *Ancylostoma caninum* (Shiramizu and Abu, 1985), and filarial worms such as *Dirofilaria immitis* (Sakamoto *et al.*, 1985) and *Litomosoides carinii* (Nakamura *et al.*, 1985).

Angiostrongylus cantonensis, which we have selected as an excellent model worm for detecting and determining the antinematode effects of drugs, was examined *in vitro* as well as *in vivo* for the mode and mechanism of action of a series of antinematode drugs including avermectin B1a and invermectin (Ishii *et al.*, 1983, 1985; Terada *et al.*, 1984). In the present study, *in vitro* effects of the new anthelmintic, milbemycin D on the motility of *A. cantonensis* and *A. costaricensis* were examined.

Materials and Methods

A. cantonensis and *A. costaricensis* were obtained from rats (Wistar strain) and mice (ddY strain) experimentally infected in our laboratory, respectively.

The isotonic transducer method previously described was used (Terada *et al.*, 1982a, 1984). The whole female worm preparation (2.5–3.0 cm) was suspended in Tyrode's solution with a tension of 0.7 to 0.8 g for *A. cantonensis* and of 0.4 g for *A. costaricensis*. In all figures showing the motility, a relaxation of the whole worm preparation is indicated by a downward deflection of the tracing recorder pen. Drugs in a single or cumulative dose were given successively at the points shown by the symbols in the figures. Preparations were kept exposed to drugs until the end of experiments or until they were washed with Tyrode's solution for about 30 min at times shown by point W in the figures.

For examining preliminarily *in vivo* effects of milbemycin D, male Wistar rats (3 months

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post infection) and male ddY mice (45 days post infection) inoculated with 20 third stage larvae each of *A. cantonensis* and *A. costaricensis*, respectively, were used. One to 14 days after a single oral treatment with milbemycin D at doses of 0.2 to 25.0 mg/kg of body weight, *in vitro* examination was done on female worms recovered.

Milbemycin D was kindly supplied by Sankyo Co. Ltd. and was suspended in a 30% glycerol solution for administration to experimental animals. The suspension of milbemycin D was diluted serially with a 0.9% NaCl solution to study its effects *in vitro*. Other drugs used were obtained from the following sources: eserine salicylate, bicuculline (Sigma); picrotoxin (Tokyokasei); strychnine sulfate, dibenamine hydrochloride (Nakarai); phentolamine mesylate (Takeda) and pyrantel tartrate (Pfizer Taito). The bicuculline was dissolved in dimethyl sulfoxide and added to the organ bath to make final concentrations of the solvent of 0.5% or less, which had little effect on the motility of *A. cantonensis*. Other drugs were dissolved in a 0.9% NaCl solution and the concentrations refer to the weight of the salts.

Results

In vitro effects of milbemycin D on the motility of adult female A. cantonensis and A. costaricensis

Milbemycin D paralyzed *A. cantonensis* at 10^{-11} g/ml and greater. Though amplitude of relaxation was not always dependent on the concentration of milbemycin D, the time required to cause a complete paralysis depended

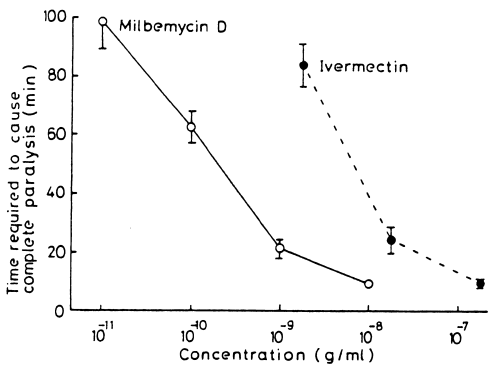

Fig. 1 Effects of milbemycin D on the motility of *A. cantonensis*: Concentration-response curve. The result on ivermectin was cited from Terada *et al.* (1984). Valures are expressed as mean ± SE (vertical bars) (n = 6–9).

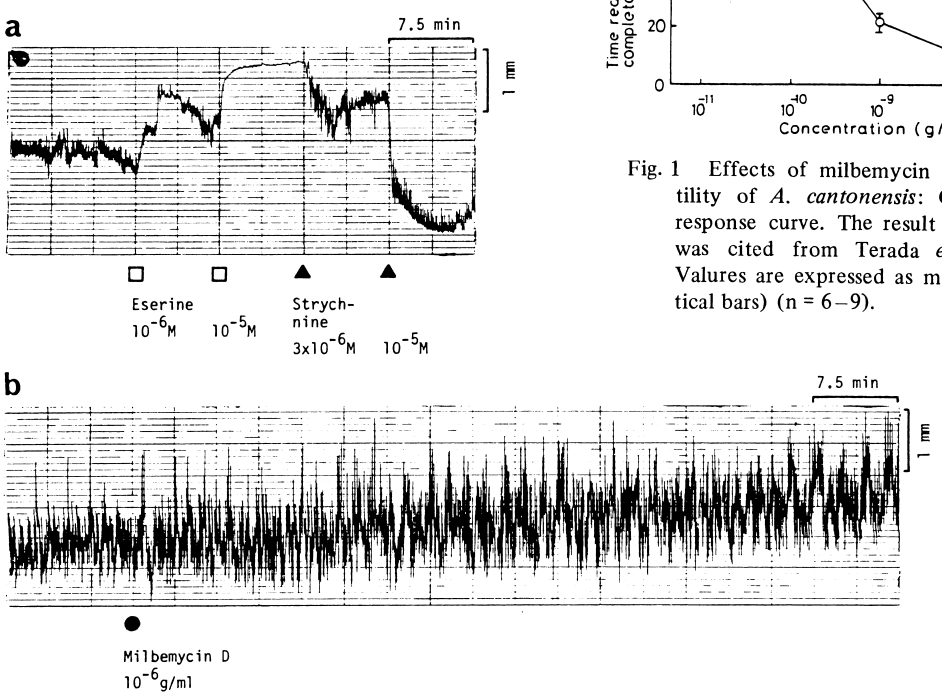

Fig. 2 Effects of eserine and strychnine (a), and milbemycin D (b) on the motility of *A. costaricensis*.

on the concentration of the drug. The time varied from 98.7 ± 9.5 min at 10^{-11} g/ml to 9.5 ± 1.1 min at 10^{-8} g/ml (Fig. 1). As cited in the figure from Terada *et al.* (1984), the *in vitro* effect of milbemycin D against *A. cantonensis* was stronger 10 times or more than that of ivermectin.

Against *A. costaricensis,* cholinergic agents like eserine (10^{-6}–10^{-5} M, an inhibitor of acethylcholinesterase activity) and strychnine (3×10^{-6}–10^{-5} M, an inhibitor of the release of acetylcholine in *A. cantonensis,* Terada *et al.,* 1982a, 1984) showed similar effects as in *A. cantonensis* (Fig. 2a). Little paralyzing effect was, however, seen when milbemycin D at even 10^{-6} g/ml was given to the preparation (Fig. 2b).

Effects of some neuropharmacological agents on the paralyzing action of milbemycin D in A. cantonensis

In a preparation paralyzed with milbemycin D (10^{-8} g/ml) this was reversed by picrotoxin (5×10^{-5}–10^{-4} M, an inhibitor of choloride ionophore), but a remarkable contraction was caused by the further addition of bicuculline (3×10^{-5} M, a GABA receptor antagonist). The preparation contracted with these gabergic antagonists was paralyzed again when strychnine (3×10^{-6} M) was given (Fig. 3a). In the preparation contracted with pyrantel (3×10^{-8} M, a cholinergic nicotinic agonist, Aubry *et al.,* 1970; Terada *et al.,* 1983), a similar paralyzing effect of milbemycin D was produced and the antagonistic effect of bicuculline was also stronger than that of picrotoxin (Fig. 3b).

Though eserine caused a sustained and strong contraction in the untreated preparation (Terada *et al.,* 1982a, b, 1984), only a transient and slight contraction was elicited by eserine (10^{-6} M) in the preparation paralyzed with milbemycin D (10^{-9} g/ml). A remarkable and sustained contraction was, however, elicited in the treated preparation after the combined application of eserine and picrotoxin (5×10^{-5} M) (Fig. 4a). In comparison with conspicuous antagonistic effects of gabergic antagonists, little influence was seen when such α-adrenergic antagonists as dibenamine (5×10^{-5} M) and phentolamine (2.8×10^{-6} M) were given to the preparation paralyzed with milbemycin D (10^{-9}–10^{-8} g/ml) (Figs. 4a, b and c).

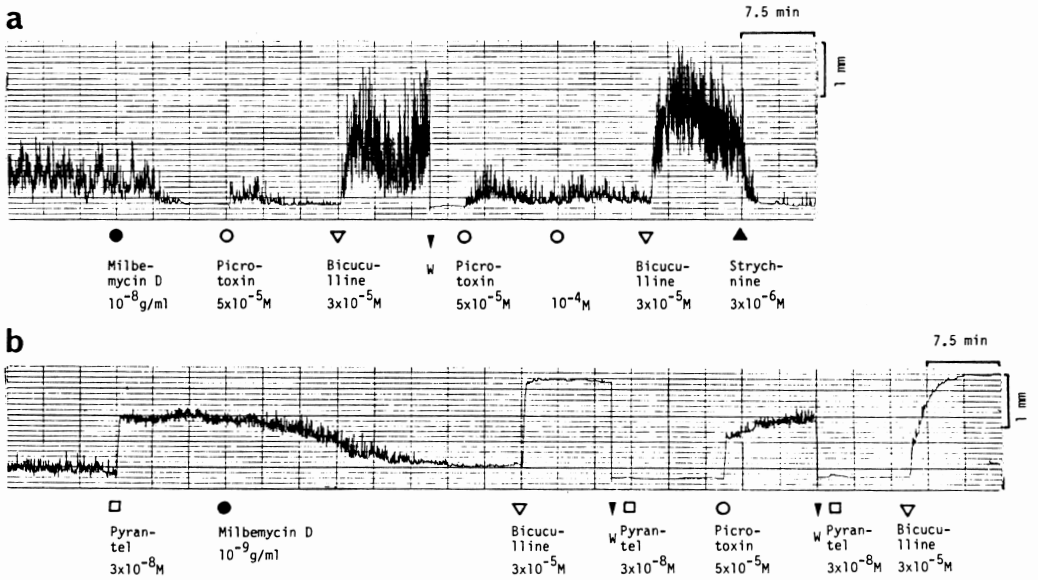

Fig. 3 Effects of gabergic antagonists on the paralyzing action of milbemycin D in *A. cantonensis.*

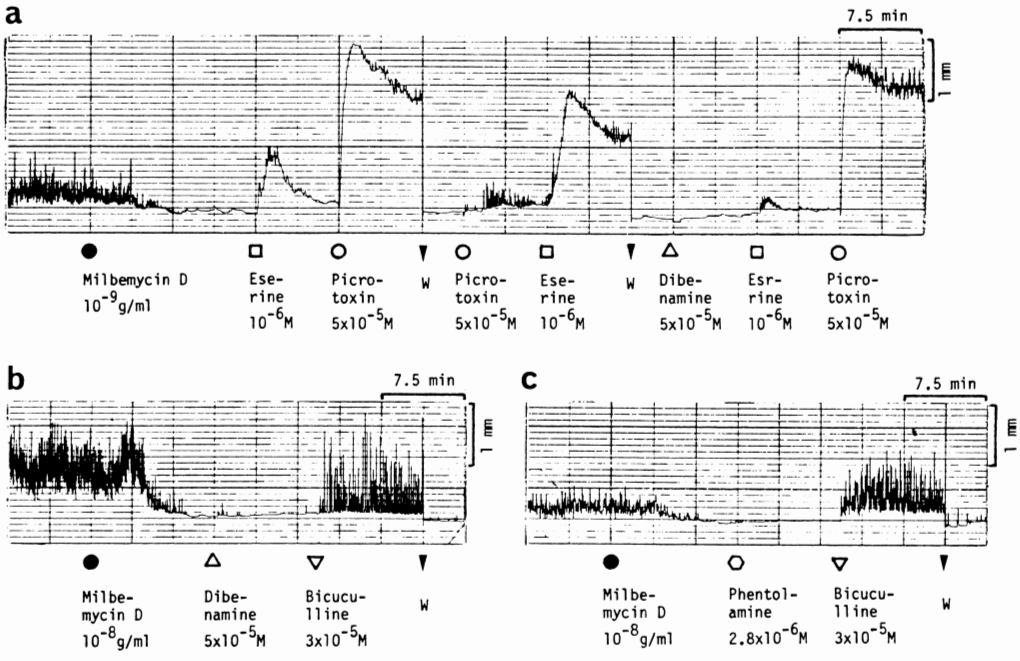

Fig. 4 Effects of some neuropharmacological agents on the paralyzing action of milbemycin D in *A. cantonensis.*

In vitro motility of adult female A. cantonensis and A. costaricensis pretreated in vivo with milbemycin D

To investigate preliminarily the reasonable doses for *in vivo* studies against *A. cantonensis* and *A. costaricensis,* a trial was undertaken with a few animals infected with each of these nematodes. *In vitro* motility was examined on adult female worms of both nematodes pretreated *in vivo* with this anthelmintic. One to 14 days after a single oral treatment with milbemycin D at doses of 0.2 to 25.0 mg/kg of body weight, female worms were isolated from the animals and their *in vitro* motility and responses to drugs such as eserine and picrotoxin were examined. From these aspects, the examined worms were divided into three groups; little influenced, slightly inhibited and markedly inhibited. As to the worms regarded as little influenced, the motility was conspicuous and eserine (10^{-6} M) caused a sustained and strong contraction, and no more effect was seen when picrotoxin (5×10^{-5} M) was given after eserine (Figs. 5a–c). Regarding the worms in the markedly inhibited group, no or only a slight motility was seen and eserine (10^{-6} M) caused a transient and slight contraction. In addition, the contraction was enhanced by picrotoxin (5×10^{-5} M) but still rather transient (Figs. 5g–i). Compared to the above two groups, milder inhibition in the motility and moderate responses to eserine and picrotoxin were seen in the worms regarded as slightly inhibited (Figs. 5d–f). On 5 to 7 female worms recovered from each animal in each treatment, the *in vitro* examination was carried out and the ratio (%) was calculated from the number of worms divided into these three groups (Fig. 6). Regarding *A. costaricensis* treated with 5.0 or 25.0 mg/kg *in vivo,* little effect was observed on the 1st and 7th day after treatment. On the other hand, against *A. cantonensis,* some effects were seen at a dose of 0.2 mg/kg and the effect was dose-dependent. At 0.2 mg/kg, 40.0 and 14.3% were slightly inhibited on the 1st and 7th day, respectively. At 1.0 mg/kg, a marked inhibition was seen only 1 day after treatment (50.0%), and the

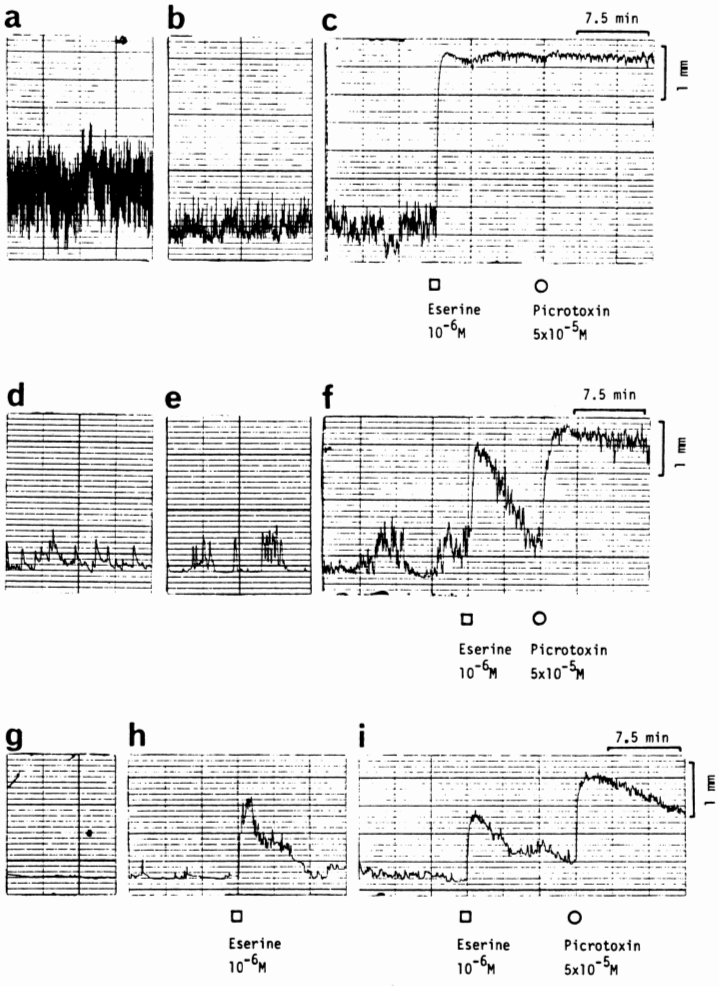

Fig. 5 *In vitro* motility and responses to eserine and pictotoxin of adult female *A. cantonensis* pretreated *in vivo* with milbemycin D. Representative tracings of little influenced (a–c), slightly inhibited (d–f) and markedly inhibited (g–i) are shown.

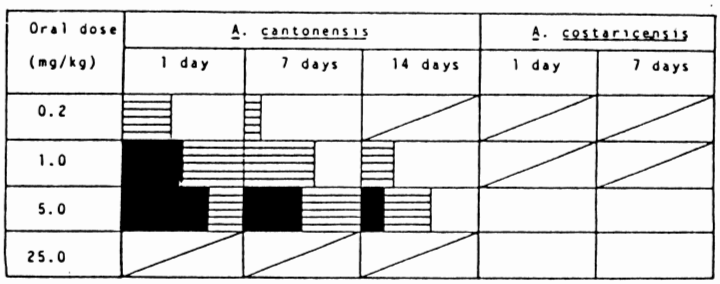

Fig. 6 *In vitro* motility and responses to eserine and picrotoxin of adult female *A. cantonensis* and *A. costaricensis* pretreated *in vivo* with milbemycin D: Ratio (%) of worms regarded as little influencet (□), slightly inhibited (▤) and markedly inhibited (■) as shown in Fig. 5.

slight inhibition was 50.0, 60.0 and 28.6% on the 1st, 7th and 14th day, respectively. At 5.0 mg/kg, a marked inhibition was seen even on 14th day, and the ratio was 71.4, 50.0 and 20.0% on the 1st, 7th and 14th day, respectively.

Discussion

It has been reported that avermectin B1a and ivermectin paralyze *Ascaris suum* and *A. cantonensis* through the gabergic mechanism (Kass *et al.*, 1980, 1982; Terada *et al.*, 1984; Campbell 1985). In our previous studies, both of the anthelmintics paralyzed *A. cantonensis* and this action was antagonized by gabergic antagonists like picrotoxin and bicuculline (Terada *et al.*, 1984). However, both these drugs as well as piperazine and γ-aminobutyric acid (GABA) had little effect on the motility of *A. costaricencis* (Sano *et al.*, 1985). In the present study, milbemycin D similarly caused a strong paralysis on *A. cantonensis* but not on *A. costaricensis*. The paralyzing effect against *A. cantonensis* was produced at 10^{-11} g/ml and greater. As a similar effect was elicited by avermectin B1a at 3.0×10^{-18} g/ml or more and by ivermectin at 2.5×10^{-9} g/ml or more (Terada *et al.*, 1984), it was suggested that milbemycin D was more effective than ivermectin against *A. cantonensis in vitro*.

As described in previous papers (Terada *et al.*, 1982a, b, 1984), paralysis in this worm could be caused by either stimulation of the gabergic and/or α-adrenergic mechanisms or by inhibition of the cholinergic mechanism. The paralyzing action of milbemycin D was antagonized by gabergic antagonists like picrotoxin and bicuculline but not by α-adrenergic antagonists as dibenamine and phentolamine. Therefore, it seems likely that milbemycin D paralyzes *A. cantonensis* through the gabergic mechanism like avermectins.

Following assumptions are described by Campbell (1985); Avermectins appear to stimulate the release of GABA and enhance the binding of GABA to its receptor, which is on the postsynaptic membrane of an excitatory motorneuron in nematodes. Rather than competing with GABA by binding to its receptor, avermectins probably bind to some other part of the GABA-receptor-ionophre complex. The enhanced GABA binding results in an enhanced flow of chloride ions into the cell with consequent hyperpolarization and elimination of signal transmission. In our previous study on the paralyzing action of avermectins on *A. cantonensis* (Terada *et al.*, 1984), the antagonistic effect of picrotoxin, an inhibitor of chloride ionophore (Andrews and Johnston, 1979) was more conspicuous than that of bicuculline, a GABA receptor antagonist (Andrews and Johnston, 1979). This may coincide well with the assumptions described above. However, in the present study on the paralyzing action of milbemycin D, antagonistic action of these two agents was inverted and bicuculline was more stronger. These results may show that there are some differences between avermectins and milbemycin D in their site or mechanism of the gabergic stimulation.

If milbemycin D is also conspicuously effective *in vivo* as well as *in vitro* against blood nematodes like *A. cantonensis*, the drug must be absorbed into blood plasma before affecting them. Therefore, it seems reasonable to investigate preliminarily whether milbemycin D is actually effective *in vivo* and also how long the duration of action is. From the results on the *in vitro* motility of adult female worms which were pretreated *in vivo* with milbemycin D, it became probable that this drug had notable effects on the motility of *A. cantonensis* at the doses of 1.0 to 5.0 mg/kg, while against *A. costaricensis* even 25.0 mg/kg of the drug had little effect. Though it was reported against intestinal nematodes including *Ancylostoma caninum* that milbemycin D had almost complete effects at 0.05 to 0.1 mg/kg (Shiramizu and Abu, 1985), we could select the doses around 5.0 mg/kg as reasonable ones for *in vivo* studies against our blood neamtodes. Thus, *in vivo* effects of milbemycin D against adult worms of these two nematodes were succes-

sively examined (Dharejo *et al.*, 1986). There was a good coincidence between the results on the *in vitro* motility and ones obtained *in vivo* on such other parameters as changes in body weight, death of host animals and recovery rate of worms. Namely, little *in vivo* effect was observed in *A. costaricensis* treated with milbemycin D of 10 successive daily doses of 25.0 mg/kg, but remarkable effects were in *A. cantonensis* treated with 5 or 10 consecutive daily doses of 5.0 mg/kg.

Summary

Effects of a new anthelmintic, milbemycin D on the motility of *A. cantonensis* and *A. costaricensis* were examined. 1) Milbemycin D paralyzed *A. cantonensis* at 10^{-11} g/ml and greater, but did not affect *A. costaricensis* even at 10^{-6} g/ml. 2) The paralyzing action of milbemycin D on *A. cantonensis* was antagonized by gabergic antagonists such as picrotoxin and bicuculline, but not by α-adrenergic antagonists like dibenamine and phentolamine. Between these bicuculline was found to be more effective than picrotoxin. In the preparation paralyzed with milbemycin D, only a slight and transient contraction was produced by eserine, but a strong and sustained contraction was caused after the combined application of eserine and picrotoxin. These results suggest that milbemycin D causes paralysis in *A. cantonensis* probably through the gabergic mechanism like avermectins. 3) *In vitro* motility was examined on adult female worms which were pretreated *in vivo* with milbemycin D. From the degree of their motility and responses to eserine and picrotoxin, it was shown that milbemycin D had little effect on *A. costaricensis* pretreated *in vivo* with a single oral dose of 25.0 mg/kg, but the drug was notably effective on *A. cantonensis* at 1.0 to 5.0 mg/kg. Thus, we could select the doses around 5.0 mg/kg to examine more reasonably *in vivo* activities of milbemycin D against these blood nematodes.

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広東住血線虫およびコスタリカ住血線虫の自動運動に及ぼす milbemycin D の作用

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広東住血線虫およびコスタリカ住血線虫の自動運動に及ぼす milbemycin D の作用を検討した．1) Milbemycin D は10^{-11} g/mℓ以上で広東住血線虫を麻痺させたが，コスタリカ住血線虫に対しては10^{-6} g／mℓでも影響を及ぼさなかった．2) 広東住血線虫に対する milbemycin D の麻痺作用は GABA 拮抗薬（picrotoxin, bicuculline）で拮抗されたが，α-アドレナリン拮抗薬（dibenamine, phentolamine）では拮抗されなかった．また，GABA拮抗薬のうち，bicuculline がより有効であった．Milbemycin D で麻痺した標本に対し，eserine はわずかな一過性収縮を生じたのみであったが，picrotoxin を併用すると著明でかなり持続性の収縮が生じた．これらの結果から，milbemycin D は avermectins と同様に GABA 機構を介して広東住血線虫を麻痺させることが示唆された．3) In vivo 下で milbemycin D の作用を受け回収された雌成虫体の自動運動を検討し，虫体の自動運動および eserine, picrotoxin に対する反応性から本薬物による影響の程度をみた．コスタリカ住血線虫は milbemycin D の25.0mg／kgの in vivo 処理では影響を受けなかったが，広東住血線虫は 1.0 ～ 5.0 mg／kgの in vivo 処理で著明な作用を受けた．これらの結果から，上記両線虫に対する milbemycin D の in vivo 効果を検討する場合には，5.0 mg／kgを中心とする用量が合理的であることが示唆された．