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A study on the mate-killer toxin by microinjection in *Paramecium*

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SUMMARY

The killing action by mu toxin, which is contained in the cytoplasm of stock 540, *Paramecium primaurelia*, was demonstrated against the various stocks of paramecia by means of microinjection. Most of the toxin is present in the soluble fraction of the host cytoplasm. The toxin was precipitated by ammonium sulphate at 50–80% saturation, and was almost completely inactivated by incubation at 60 °C for 30 min. Pre-autogamous paramecia were more sensitive than post-autogamous ones to the toxin. Paramecia which bear endosymbionts were generally resistant to the mate-killer toxin.

1. INTRODUCTION

Endosymbionts of the *Paramecium aurelia* complex have been investigated extensively by many workers since killers and sensitives were discovered by Sonneborn (1938) (see review by Preer, Preer & Jurand, 1974). Most killer paramecia liberate toxic particles, such as kappa and lambda, into the medium in which they live. Preer & Preer (1964) showed that isolated R bodies of stock 7 of *P. biaurelia* contain most of the killing activity of the whole bright kappa. Further, Preer & Preer (1967) suggested that the killing toxin is a protein found on the R body ribbon. On the other hand, mate-killers must act in a very different way. Siegel (1953) discovered mate-killers which contain mu particles in stock 138 of *P. octaurelia*. Mate-killers cause their sensitive partners to die after conjugation, but unlike kappa liberate no toxin into the medium in which they live. Cell to cell contact, but not nuclear transfer, is necessary for mate-killing. Mate-killers in other stocks of *P. octaurelia*, in several stocks of *P. primaurelia* and stock 570 of *P. biaurelia* have been investigated by Levine (1953), Beale & Jurand (1960, 1966), Gibson & Beale (1961) and Preer *et al.* (1972). However, these workers were mainly concerned with general characteristics of mu and with genic control of the symbionts.

So far, no information about the toxin produced by mate-killer symbionts has been obtained. The present paper deals with the localization and the nature of mate-killer toxin. The study has been carried out by means of the technique of micro-injection.

2. MATERIALS AND METHODS

Stocks and culture methods. The various killer and sensitive stocks of the *Paramecium aurelia* complex used in this study were kindly supplied by Drs Sonneborn and Preer at Indiana University, U.S.A. Details of these stocks are given by Preer *et al.* (1974). The culture medium used was a 0·25 % infusion of dried Scottish grass buffered with one gram of $Na_2HPO_4.12H_2O$/l and inoculated with *Klebsiella aerogenes* 1–2 days before use. Stock 540 mate-killer of *P. primaurelia* was cultivated at 0·5 fission per day, and produced long forms of mu. Other killer cultures used as recipients were maintained at 27 °C and grown at appropriate fission rates. Before use, killer paramecia were examined for the presence of symbionts by staining with lacto-orcein (Beale & Jurand, 1966).

Materials for injection. Mu particles of stock 540 were purified by the method described by Sonneborn (1970). Purified mu was suspended in the same volume of Dryl's solution as that of packed cells. Pressed juice from 540 cells bearing mu symbionts was prepared as follows. One ml of packed paramecia were obtained from 15 to 20 l. of culture. After removing the culture fluid by repeated centrifugation at 3000 rev/min the cells were covered with mineral oil and centrifuged for 20 min at 40000 rev/min in a refrigerated centrifuge. The supernatant liquid, or pressed juice, contained the soluble materials from the paramecia which were crushed by the very high centrifugal force. It was removed and stored in a refrigerator for a few days. Pressed juice yielded about 0·3 ml from 1 ml of packed cells. Pressed juice from 570 mu cytoplasm of *P. biaurelia* was prepared in the same way. The various ammonium sulphate fractions obtained by adding saturated solution to the pressed juice were dissolved in the same volume of Dryl's solution as that of pressed juice and dialysed against distilled water for 2 h and then against Dryl's solution for 1 h in a refrigerator.

Microinjection. Injection was carried out by the method of Koizumi (1974). Approximately 5000 μ^3 of cytoplasm, mu particle suspension, pressed juice or ammonium sulphate fraction dissolved in Dryl's solution were injected into paramecia at the following stages: 'post-autogamous' (vegetative) and 'pre-autogamous' (autogamy being induced immediately after injection). Post-autogamous paramecia are those which have passed through autogamy and then undergone 4 or 5 fissions. These animals do not go into autogamy again for a considerable number of fissions. Pre-autogamous paramecia are those which have undergone 15–20 fissions in the stocks of *P. tetraurelia* or 40–45 fissions in the stocks of *P. primaurelia* since the previous autogamy and are therefore likely to enter autogamy once again when starved. Autogamy was induced by starvation of pre-autogamous animals immediately after injection.

3. RESULTS

Following injection, animals were left in Dryl's solution or exhausted medium (Sonneborn, 1950) for 1 day in the case of the post-autogamous animals and 2 days in the pre-autogamous ones to induce autogamy, and then grown at 27 °C. Paramecia, if killed after injection, either died without undergoing fission or after 1–3

fissions, occasionally 4–5 fissions, in 1–3 days. Some animals among the survivors were apparently affected by injection but could recover from the slow growth after several days.

Injection of 540 cytoplasm which bore particles into 51 and 540 sensitives caused toxic action against both pre-autogamous and post-autogamous recipient animals (see Table 1). From the results on particles and pressed juice given in Table 1, it is

Table 1. *Toxic action of materials obtained from 540 mate-killer animals against 51 and 540 sensitives*

	% of animals killed			
	51		540	
Material injected	Pre-autogamous	Post-autogamous	Pre-autogamous	Post-autogamous
Cytoplasm	65 (13/20)	70 (14/20)	66 (25/38)	60 (12/20)
Particles (mu)	7 (3/45)	0 (0/20)	4 (1/25)	6 (2/33)
Pressed juice	50 (22/44)	35 (12/32)	83 (20/24)	17 (4/24)
Pressed juice (51 killer)	0 (0/22)	0 (0/20)	0 (0/20)	0 (0/20)

concluded that the killing toxin is present in a soluble fraction of the cytoplasm rather than contained in purified mu particles. In fact, there was no killing action by mu particles against 51 in vegetative animals and only seven per cent in pre-autogamous ones. Similar results were also obtained in the case of both stages of 540 animals in which only 7 % of the recipients were killed. On the other hand injection of pressed juice into 540 paramecia caused killing of as many as 83 % of the pre-autogamous animals. Control experiments in which pressed juice prepared from 51 sensitive and killer animals was injected did not show any killing action against 51 and 540 at either stage.

The toxic action of pressed juice from 540 killer cytoplasm against various killer and sensitive animals is summarized in Table 2. The pressed juice was capable of killing vegetative-stage animals of all the sensitive stocks used here. On the other hand, killer animals which bear symbionts were generally resistant to the toxin in the pressed juice, except for 7 kappa killer in which 24 % of the recipients were killed. Thus the resistance to the toxin was not always associated with the presence of mu symbionts, except for pi, and was also found in those animals which bear other symbionts. Furthermore, no toxic action was shown against pre-autogamous cells in 51 and 540 killer stocks.

In order to investigate the nature of the toxin, ammonium sulphate precipitates from pressed juice were tested for killing activity against stock 51 sensitives. Most of the killing activity was found to be contained in the 50–80 % ammonium sulphate fraction as shown in Table 3. It was also demonstrated that the fraction obtained here did not manifest any killing action against 51 killer animals.

The killing action of both pressed juice and the 50–80 % ammonium sulphate fraction against pre-autogamous animals of both 51 and 540 was reduced to almost

Table 2. *Toxic action of the pressed juice from 540 killer cytoplasm against various killer and sensitive animals in the vegetative stage*

Killer				Sensitive			
Species	Stock	% of animals killed		Syngen	Stock	% of animals killed	
P. primaurelia	540 mu	0 (0/25)	0* (0/24)	*P. primaurelia*	540	17 (4/23)	83* (19/23)
P. primaurelia	551 mu	9 (3/33)		*P. primaurelia*	513	15 (4/27)	85* (17/20)
P. biaurelia	570 mu	3 (1/31)		*P. primaurelia*	16	41 (9/22)	
P. biaurelia	7 kappa	24 (11/47)		*P. biaurelia*	7	41 (9/22)	
P. biaurelia	249 kappa	8 (2/25)		*P. triaurelia*	152	53 (11/21)	
P. biaurelia	562 kappa	0 (0/20)		*P. tetraurelia*	51	35 (12/32)	
P. biaurelia	1010 nu	0 (0/24)		*P. tetraurelia*	51 pi	20 (4/20)	
P. tetraurelia	51 kappa	0 (0/24)	0* (0/24)	*P. octaurelia*	138	9 (2/24)	36* (8/22)
P. tetraurelia	51 ml kappa	5 (1/24)					
P. octaurelia	299 lambda	0 (0/21)		Syngen 3 (*P. caudatum*)	Ksy	78 (17/22)	
P. octaurelia	214 gamma	0 (0/22)					

Asterisks indicate pre-autogamous animals in which autogamy was induced after injection.

Table 3. *Toxic action of ammonium sulphate fractions obtained from pressed juice of 540 mate-killer cytoplasm against 51 animals*

Ammonium sulphate fraction (% of saturation)	% of animals killed		
	Sensitive		Killer
	Pre-autogamous	Post-autogamous	Post-autogamous
0–40	0 (0/20)	—	—
40–50	13 (3/24)	4 (1/24)	—
50–80	48 (11/23)	17 (4/23)	0 (0/24)
80–95	4 (1/23)	—	—
Pressed juice	50 (22/44)	29 (6/21)	0 (0/24)
Ammonium sulphate* fraction (95%)	4 (1/25)	0 (0/32)	0 (0/29)

* Prepared with pressed juice from 51 killer cytoplasm.
— Not tested.

half by freezing with liquid nitrogen. Such samples no longer showed killing action against vegetative animals.

Heat stability of the toxin in the fractions was examined by incubating them in a water bath at 60 °C. Each ampoule of material was chilled in an ice bath after 5, 30 and 60 min of incubation respectively, and then injected into pre-autogamous stage of 51 sensitives. The killing action was determined by injection of these materials and was shown to be as follows: untreated 54% (12/22); 5 min 22% (5/23); 30 min 4% (1/23); 60 min 0% (0/21), respectively. The activity of the toxin was, therefore, reduced by more than 90% after incubating at 60 °C for 30 min.

Injection of pressed juice from 570 mate-killer cytoplasm into pre-autogamous animals of both 51 and 540 also caused killing action. The rate was 78% (15/19) against 51, 46% (11/24) against 540, and 0 (0/20) against 570 vegetative killer animals.

4. DISCUSSION

Mate-killer action by mu in any way other than cell to cell contact at conjugation has never been found (Preer *et al.* 1974). One exception was, however, found with *in vitro* cultured mu in which 138 mu kills lambda free 299 paramecia (Williams, 1971). In the present paper it is reported for the first time that paramecia can be killed by microinjection of extracts of mu killers without cell to cell contact. It seems to be clear that the mate-killer toxin is present in the soluble fraction of the host cyto plasm rather than in the mu symbionts themselves. This fact suggests that mu toxin is a so-called exotoxin.

From the results on precipitation with ammonium sulphate, heat stability and the decrease of toxic activity by freezing-thawing it may be suggested that the mate-killer toxin is protein.

Injection of kappa and lambda into sensitive paramecia never caused killing action against recipient animals (Koizumi, 1974, and unpublished data), hence the

killing action of kappa and lambda is due to being taken in by mouth, as is well known. Further, an electron microscopic study of the effects of isolated stock 7 kappa on sensitives by Jurand, Rudman & Preer (1971) suggested that the primary target of the kappa toxin is the food vacuole membrane. On the other hand, Siegel (1954) suggested that the mu of 138 acts on the nuclei. In the present paper, pre-autogamous recipient paramecia in which autogamy was induced immediately after injection, were much more sensitive to the toxin than post-autogamous animals. Similar results were obtained by Koizumi & Kobayashi (unpublished) in experiments on conjugating paramecia. These results suggest that nuclei in the process of nuclear reorganization are more sensitive to the toxin than are nuclei in the vegetative stage. However, no cytological evidence on the specific target has been obtained. Nevertheless, it is important to note that the mate-killer toxin is able to kill vegetative paramecia.

It is most remarkable that paramecia bearing symbionts other than mu are generally resistant to the toxin. Stock 7 kappa killers are an exception. Furthermore, the resistance is not always associated with killing particles. Thus resistance was found in paramecia which bear non-toxic particles such as 1010 mu, but not pi. So far, the mechanism of protection to the mu toxin in those animals is unknown.

No toxic action was revealed by pressed juice from the cytoplasm of 51 killer. Kappa toxin is, therefore, entirely associated with R bodies. Pressed juice from mate-killer stock 570, which bears mu with R bodies like kappa, showed strong killing action against 51 and 540 pre-autogamous animals, but not against 570 mate-killers. These facts suggest that a primary difference in the toxic actions of mu and kappa is whether the toxin is liberated into the host cytoplasm or not.

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