ZOOLOGICAL SCIENCE 6: 691-696 (1989)

Synergistic Effects of Calyx Fluid and Venom of *Apanteles kariyai* WATANABE (Hymenoptera:Braconidae) on the Granular Cells of *Pseudaletia separata* WALKER (Lepidoptera:Noctuidae)

HARUHISA WAGO and TOSHIHARU TANAKA[1]

Department of Bacteriology, Saitama Medical School, Moroyama 350-04, Japan, and [1]Department of Entomology, Texas A&M University, College Station, Texas 77843, USA

ABSTRACT—This study describes some of the effects of calyx and venom fluids of *Apanteles kariyai* on the morphology and the filopodial function of *Pseudaletia separata* granular cells, using scanning electron microscopy and an *in vitro* incubation system. Calyx fluid contained virus-like particles, which were shown to be round or oval with a smooth surface and a size of about 225 nm in diameter under the scanning electron microscopy. When granular cells were incubated in the presence of either calyx or venom fluids, their filopodia elongated extensively at the cell periphery just like the control. However, incubation of cells with both calyx and venom fluids greatly inhibited filopodial elongation and further caused a remarkable cytolysis during the incubation time. Calyx fluid seems to require components of the venom fluid to have inhibitory effects on the initial cellular defence reaction of host larvae. In this paper, the possible roles of calyx and venom fluids in suppression of the hemocytic reaction of host will be discussed.

INTRODUCTION

Eggs and larvae of endoparasitoids normally complete development in the hemocoel of suitable hosts without triggering host cellular defence reactions such as encapsulation. In braconid parasitoids, calyx and venom fluids injected with eggs during oviposition and teratocytes derived from parasitoid serosal cells seem to be responsible for inhibition of host encapsulation. In the egg stage of parasitoid, the surface coat of egg [1], the calyx fluid and venom (alone or together) are apparently essential in evading the host defence system [2, 3], whereas in the larval stage teratocyte-derived substances in conjunction with calyx and venom fluids are involved in inhibition of hemocytic encapsulation [4]. However, the mechanism through which these inhibitory factors prevent the encapsulation of branonid parasitoids is unknown. In *A. kariyai*, the eggs evade the cellular encapsulation through the activity of calyx and venom fluids and a coating of egg surface with venom fluid is important to depress the nonself recognition capacity of host hemocytes [3]. In addition, prevention of nonself recognition was suggested to be due to the inhibition of filopodial elongation of granular cells [5]. Since filopodial elongation of cells is an essential factor in the progress of cellular reactions such as phagocytosis or encapsulation [6], this study was undertaken to examine the effects of calyx fluid and venom of *A. kariyai* on the surface ultrastructure and filopodial function of granular cells of *P. separata* larvae using an *in vitro* incubation system.

Accepted November 9, 1988
Received August 31, 1988

MATERIALS AND METHODS

Insect cultures The braconid parasitoid, *Apanteles kariyai* was laboratory-reared on larvae of *Pseudaletia separata* maintained on the artificial medium described by Hattori and Atsusawa [7] at 25±1°C under a 16 hr light-8 hr dark photo-

periodic regime and fed a 30% sugar solution in the glass tube [3]. Host larvae were individually parasitized to avoid the superparasitism. About 100 host larvae were maintained in 200 ml flask under aseptic conditions until the fifth instar, then a group of 20–30 host larvae were separately reared in $15 \times 20 \times 5$ cm plastic case.

Collection of the hemocyte suspension from host larvae Hemolymph of 20 individuals of day 2-sixth instar larvae of *P. separata* was collected in a chilled small tube containing 1.5ml of insect physiological saline (IPS; 150 mM NaCl, 5 mM KCl) with 0.1% phenylthiourea (PTU), and centrifuged at 600 rpm for 5 min at 4°C to obtain the hemocytes. After twice rinse of hemocytes in IPS at 4°C, hemocyte concentration was finally adjusted to 1×10^6 cells/ml with IPS. Observation of hemocyte suspension with 0.5% neutral red stain solution showed that the granular cells made up about 42.5% of the total hemocyte count.

Collection of calyx and venom fluid Calyx fluid and venom were individually collected from lateral oviduct and venom reservoir of female wasp using a binocular microscope as described previously [8]. Briefly, venom fluid was first collected by rupturing venom reservoirs in 3 μl of IPS. Second, calyx fluid was collected from the lateral oviduct in 3 μl of IPS and then centrifuged at 800 rpm for 3 min to remove eggs and cell debris. Since two microliter (1/3 female wasp equivalent for each) of their fluid is effective in *in vivo* inhibition of encapsulation ability against parasitoid eggs, this concentration was also used to treat the granular cells.

Treatment of incubated granular cells with the calyx fluid and venom First, hemocyte suspension collected as mentioned above was similarly spun and resuspended in a equal volume of cell-free plasma, which had been already collected in a cold-tube containing a few crystals of PTU. Hemocyte suspension (0.15 ml) was incubated at 4°C for 60 min to allow the granular cells to attach on a glass surface of 7×7 mm coverslip in each well of Lab-Tex 8-chamber slide, because a 4°C temperature inhibits the filopodial elongation of granular cells [9]. Thereafter, supernatant plasma was removed and gently rinsed with chilled IPS three times. Fifty μl of each solution of venom, calyx fluid, and a 1:1 mixture of calyx and venom fluids was respectively overlayered on the hemocyte monolayers, and further incubated at 25°C for 120 min. The cell viability at the onset of incubation was over 98% with 0.25% trypan blue. Following a 60- and 120-min incubation, the granular cells were processed as a scanning electron microscopic preparation for the observation of the surface ultrastructure.

Scanning electron microscopy (SEM) observation After a series of procedures for SEM, including fixation, dehydration in ethanol, critical point drying, and coating with platinum, which is the case with the previous method [10], the cell preparations were examined with the SEM (Hitachi S-550) at 20 kV to observe the surface ultrastructure of the granular cells and the calyx particles.

RESULTS AND DISCUSSION

Although it had been already shown by transmission electron microscopic observation that calyx fluid of *A. kariyai* contains a lot of particles, of which each envelope had several electron-dense cores [5], the shape of envelop was unclear. SEM observation showed that a calyx particle was round or oval with a smooth surface and size of about 225 nm in diameter (Fig. 1). Furthermore, attachment on the surface of granular cells seems to suggest that virus-like particles penetrate into the cell [11] and have some effect on the granular cell function.

FIG. 1. Calyx particles under the SEM observation. Calyx particle was round or oval with a smooth surface and size of about 225 nm in diameter.

Granular cells are involved in the cellular reactions including phagocytosis and encapsulation in *P. separata* [5] like other lepidopterans [12], and possess many filopodia on the cell surface. Additionally, filopodial elongation of the cells are required for the progress of the initial cellular reactions against foreign substances [10, 13]. On the other hand, it is reported that both parasitization and manual injection of calyx and venom fluids of *Microplitis mediator* to host *P. separata* strongly suppress the filopodial elongation [5]. Conversely, although in the hemolymph of hosts parasitized by *A. kariyai,* suppression of filopodial elongation of granular cell was not evident, Sephadex particle encapsulation in the parasitized host was inhibited compared to controls [5]. In this study, degree of suppression of filopodium was expressed quantitatively by counting the number of elongated filopodia on the granular cell. Inhibitory effect of calyx and venom fluids on filopodial elongation, which was not apparent *in vivo* system, was observed *in vitro* system, although it was unclear whether concentration of calyx and venom fluids used was suitable or not. Neither calyx fluid nor venom inhibited the filopodial elongation as compared to the control (Fig. 2). Moreover, most of granular cells attached to the coverslip with more than 20 filopodia extensively elongated (Fig. 3a, b) just as control (Fig. 3d). Conversely, elongation was remarkably inhibited in the presence of both calyx and venom fluids after a 60-min incubation (Figs. 2 and 3c). The cells were unable to elongate their filopodia on the surface at the periphery. Additionally, after a 60-min treatment of cells with calyx and venom fluids mixtures, about 4.8% of attached granular cells showed a great degree of cytolysis. As shown in Figure 4b and 4c, many granules in the cytoplasm were exposed outside and in the case of more damaged cells, only a sticky cytoskelton component was observed. After a 120-min incubation in calyx and venom fluids, about 76% of cells showed a cellular damage. These results strongly suggested that the coexistence of calyx and venom fluids was important in affecting the host cellular reactions by the granular cells in *A. kariyai-P. separata* system.

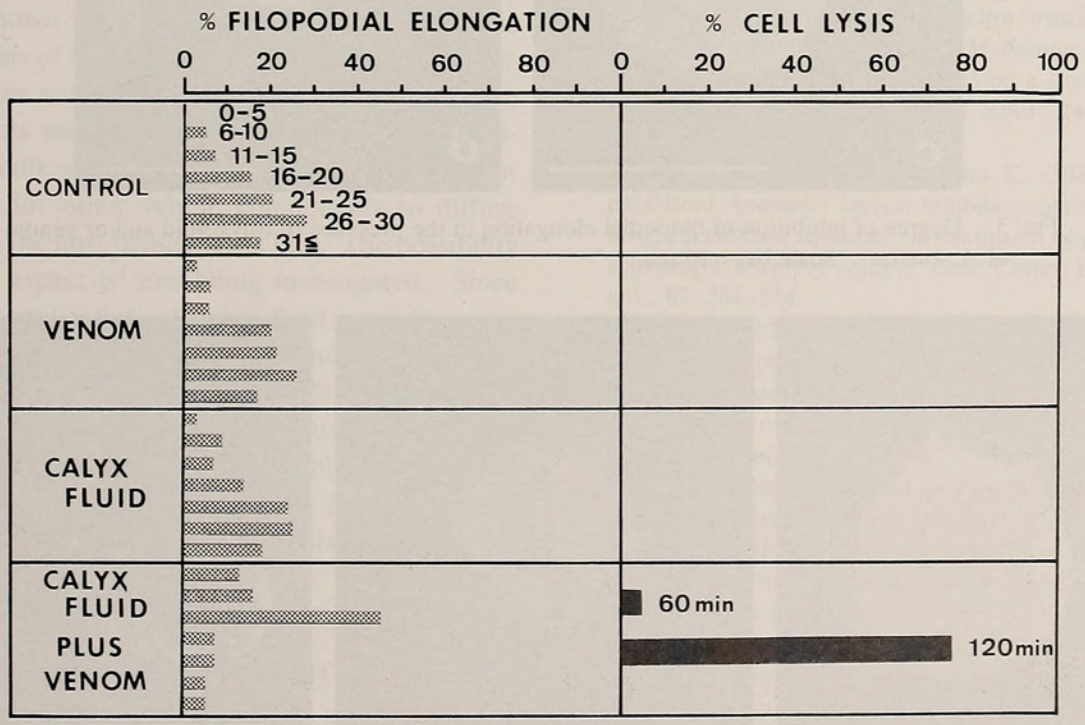

FIG. 2. Inhibition of filopodial elongation and the cytolysis of granular cells of *Pseudaletia separata* by the calyx fluid and/or venom of *Apanteles kariyai.* Granular cells of last instar host were attached on the cover-slip at 4°C for 60-min incubation. Fifty μl of each solution of venom, calyx fluid, and a 1:1 mixture of calyx fluid and venom was respectively overlayed on the hemocyte monolayers. Then, filopodial elongation was examined under the SEM after a 60-min treatment, and cytolysis was also observed after a 60- and 120-min incubation.

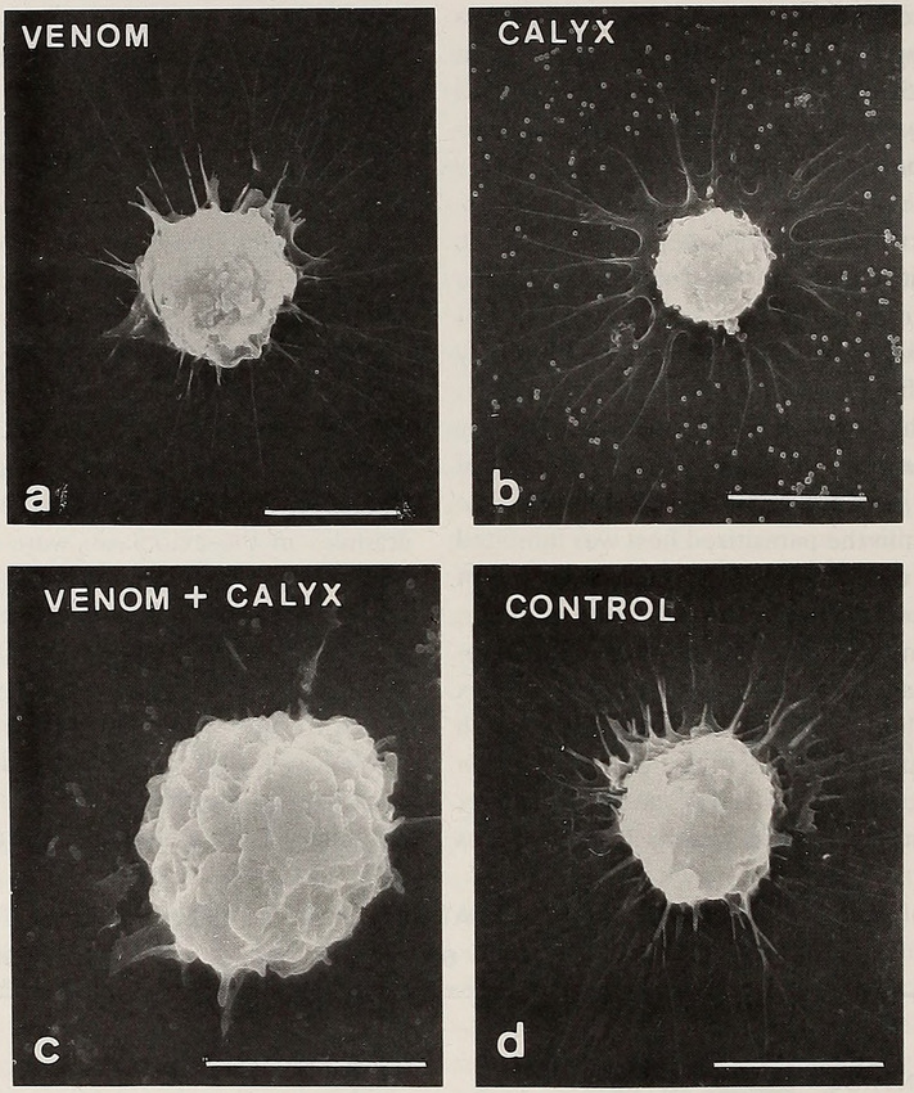

FIG. 3. Degree of inhibition of filopodial elongation in the presence of calyx fluid and/or venom of *A. kariyai*. Scale bar = 10 μm.

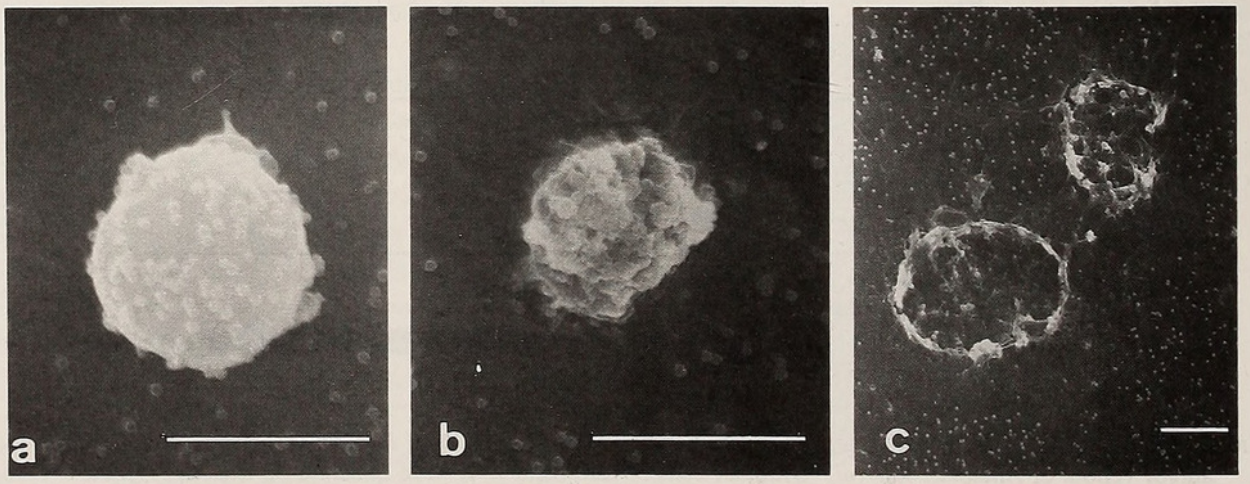

FIG. 4. Process of cytolysis of granular cells overlayed with calyx fluid and venom of *A. kariyai*. Filopodial elongation was, first of all, inhibited (a), then the intracellular granules were exposed outside (b), and finally the cytoskeltal components were only observed (c). Scale bar = 10μm.

Rizki and Rizki [14] demostrated that lamellocytes underwent a great morphological change and lost their adhesiveness in *Drosophila melanogaster* parasitized by *Leptopilina heterotoma.* These changes were induced by lamellocin contained in an accessary gland of the female reproductive system. Calyx and venom fluids also seem to give hemocytes an damage so that parasitoid eggs and larvae are not recognized as non-self material. Moreover, plasmatocytes of *Heliothis virescens* have recently been reported to be functionally interfered by the calyx virus of parasitoid *Campoletis sonorensis* [15]. Calyx fluid injection caused a removal of approximate 75% of the circulating plasmatocytes [16]. Stoltz *et al.* [17] reported that polydnavirus from braconid wasp *Cotesia melanoscela,* required venom to penetrate into the host cells. In this sense, if venom which braconid parasitoid should possess had the similar composition to a wasp venom in the family of Vespidae [18] containing an enzyme such as phospholipase A_2 or hyaluronidase, its composition could influence the membrane of hemocytes and facilitate the introduction of calyx virus into the cytoplasm. It has been known that phospholipase A_2 catalyzes the hydrolysis of structural phospholipids via synergistic action with melittin [19], and hyaluronidase hydrolizes mucopolysaccharide polymers consisting the bulk of animal connective tissue and opens passage for other venom components to diffuse through the host tissue matrix [20]. The possibility of such aspect is now being investigated. Since neither application of calyx fluid nor of venom alone induced the cytolysis of granular cells, virus-like particles of calyx fluid seems to require venom activity to alter granular cell function. In *A. kariyai,* it is unclear whether or not the number of granular cells, *in vivo,* decreases following parastization or calyx-injection. This point is also under currently investigation.

ACKNOWLEDGMENTS

We thank Dr. B. A. Webb of Texas A & M University for critical reading of the manuscript.

REFERENCES

1 Davies, D. H. and Vinson S. B. (1986) Passive evasion by eggs of braconid parasitoid *Cardiochiles nigriseps* of encapsulation *in vitro* by haemocytes of host *Heliothis virescens.* Possible role for fibrous layer in immunity. J. Insect Physiol., **32**: 1003–1010.

2 Kitano, H. (1982) Effect of the venom of gregarious parasitoid *Apanteles glomeratus* on its hemocytic encapsulation by the host, *Pieris.* J. Invertebr. Pathol., **40**: 61–67.

3 Tanaka, T. (1987) Effect of the venom of the endoparasitoid, *Apanteles kariyai* Watanabe, on the cellular defence reaction of the host, *Pseudaletia separata* Walker. J. Insect Physiol., **33**: 413–420.

4 Tanaka, T. and Wago, H. (1989) Ultrastructural and functional maturation of teratocytes of *Apanteles kariyai.* Arch. Insect Biochem. Physiol., (in press)

5 Tanaka, T. (1987) Morphological changes in haemocytes of the host, *Pseudaletia separata,* parasitized by *Microplitis mediator* or *Apanteles kariyai.* Dev. Comp. Immunol., **11**: 57–67.

6 Wago, H. (1983) Cellular recognition of foreign materials by *Bombyx mori* phagocytes: II. Role of hemolymph and phagocyte filopodia in the cellular reactions. Dev. Comp. Immunol., **7**: 199–208.

7 Hattori, M. and Atsusawa, S. (1980) Mass-rearing of the cabbage armyworm, *Mamestra brassicae* Linne and the common armyworm, *Mythimna separata* Walker (Lepidoptera: Noctuidae) on a simple artificial diet. Jap. J. Appl. Entomol. Zool., **24**: 36–38. (In Japanese)

8 Tanaka, T., Agui, N. and Hiruma, K. (1987) The parasitoid *Apanteles kariyai* inhibits pupation of its host, *Pseudaletia separata,* via disruption of prothoracicotropic hormone release. Gen. Comp. Endocrinol., **67**: 364–374.

9 Wago, H. (1982) Regulatory effects of temperature and hemolymph on inhibition and enhancement of attachment and filopodial function of phagocytic granular cells of the silkworm, *Bombyx mori.* Dev. Comp. Immunol., **6**: 231–241.

10 Wago, H. (1980) Humoral factors promoting the adhesive properties of the granular cells and plasmatocytes of the silkworm, *Bombyx mori* and their possible role in the initial cellular reactions. Cell Immunol., **54**: 155–169.

11 Stoltz, D. B. and Faulkner, G. (1978) Apparent replication of an unusual virus-like particle in both a parasitoid wasp and its host. Can. J. Microviol., **24**: 1509–1514.

12 Wago, H. (1982) Cellular recognition of foreign materials by *Bombyx mori* phagocytes:I. Immunocompetent cells. Dev. Comp. Immunol., **6**: 591–599.

13 Wago, H. (1984) *In vitro* evidence for the requirement of filopodial elongation for the progress of phagocytosis by phagocytic granular cells of the silkworm, *Bombyx mori*. Dev. Comp. Immunol., **8**: 7–14.

14 Rizki, R. M. and Rizki, T. M. (1984) Selective destruction of a host blood cell type by a parasitoid wasp. Proc. Natl. Acad. Sci. USA, **81**: 6154–6158.

15 Davies, D. H. and Vinson, S. B. (1988) Interference with function of plasmatocytes of *Heliotis virescens in vivo* by calyx fluid of the parasitoid *Campoletis sonorensis*. Cell Tissue Res., **251**: 467–475.

16 Davies, D. H., Strand. M. R. and Vinson, S. B. (1987) Changes in differential haemocyte count and *in vitro* behaviour of plasmatocytes from host *Heliothis virescens* caused by *Campoletis sonolensis* polydnavirus. J. Insect Physiol., **33**: 143–153.

17 Stoltz, D. B., Belland, E. R., Lucarotti, C. J. and MacKinnon, E. A. (1988) Venom promotes uncoating *in vitro* and persistence *in vivo* of DNA from a braconid polydnavirus. J. gen. Virol., **69**: 903–907.

18 Blum, M. S. (1981) Proteinaceous Venoms. In "Chemical Defenses of Arthoropods". Ed. by M. S. Blum, Academic Press, New York, pp. 288–328.

19 O'Conner, R. and Peck, M. L. (1978) Venom of Apidae. In "Handbook of Experimental Pharmacology, 48. Arthoropod Venoms". Ed. by S. Bettini, Springer-Verlag, Berlin, pp. 613–659.

20 Schmidt, J. O. (1982) Biochemistry of insect venom. Ann. Rev. Entomol., **27**: 339–368.