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Original Communication

Meteorus pulchricornis (Wesmael) (Hymenoptera, Braconidae) teratocytes release Mp19 protein in MpVLP, suppressing the function of hyper-spreading hemocytes in *Mythimna separata*

Yoshiaki Kato[1], Reina Sato, Takeshi Sano, Yutaka Nakamatsu[2], Ken Miura and Toshiharu Tanaka[3,*]

Laboratory of Applied Entomology, Graduate School of Bioagricultural Sciences,
Nagoya University, Nagoya, Japan.

ABSTRACT

Insects rely on their immune defense system for protection against foreign substances invading the host hemocoel. The humoral and cellular immune systems have a well-evolved relationship. The cellular immune response, encompassing processes such as encapsulation and nodule formation, is associated with local melanin formation on the surface of the foreign substance. Hemocytes identified as the hyper-spread cells (HSCs) of *Mythimna separata* larvae deposit melanin around their peripheral edge. Some granulocytes carrying pro-phenoloxydase settle near to the HSCs and may collaborate with them in melanin formation. The HSC population serves as a signal marker on the foreign substance to other hemocytes involved in encapsulation. In the present study, we examined the proteins associated with inhibiting the adhesion and extension of HSCs on foreign substances, focusing on the elimination of HSCs from the parasitized host. The *Meteorus pulchricornis* (Wesmael) (Hymenoptera, Braconidae) wasp produces virus-like particles (VLPs) consisting of proteinaceous substances, and injects them with its egg into the host hemocoel. The VLPs regulate the host cellular immune system to facilitate the development of the parasitoid eggs and larvae. We found that Mp18 and Mp19 proteins (mainly Mp19) contained in the *M. pulchricornis* virus-like particle (MpVLP) led to the inhibition of actin formation and filopodia extension in adhesive hemocytes such as granulocytes and plasmatocytes. The same protein identified with anti-Mp19 antibody was released from teratocytes at a later developmental stage. In addition, a parasitoid larva in the late stages of development expressed the same Mp19 gene, whereas smaller amount of Mp18 mRNA was observed. This result indicates that during the larval stage, the teratocytes and larvae express the Mp19 gene without temporal overlap; the protein makes the HSC and the related hemocytes inactive. This is supported by the existence of small particles around teratocytes and host tissues stained with anti-Mp19 antibody from 7 to 8 d after parasitization.

KEYWORDS: teratocytes, virus-like particle, teratocyte-secreted protein, venom gland protein

INTRODUCTION

Insects have an immune defense system against any foreign substances that penetrate the hemocoel [1-6]. The immune system responses can be generally grouped into two defense systems, namely humoral [7] and cellular [8, 9]. The humoral defense system consists of a peptide or protein response involving many cascades [10-14], producing antibacterial substances *via* the Toll and/or IMD (immune deficiency) pathway [15-18], or melanin formation

*Corresponding author: totanaka@agr.nagoya-u.ac.jp
Present address: [1]BASF Japan Agricultural Station, Tahara, Aichi Prefecture, Japan;
[2]Kogakukan University, Ise, Mie Prefecture, Japan.
[3]Nagoya University Cambodia Satellite Campus at Royal University of Agriculture, Cambodia.

around the foreign body through the phenoloxidase cascade involving plasma and hemocyte proteins [12, 19-23]. On the other hand, the cellular defense system involves phagocytosis of substances, such as bacteria, which are smaller than the hemocytes, and nodule formation in response to the large numbers of bacteria that invade the hemocoel at the same time; this involves the recognition of microbial compounds such as β-1,3-glucan, lipopolysaccharides, and peptidoglycan [6, 24-27]. Foreign substances larger than the hemocytes, such as parasitoids, are removed by encapsulation, which involves the enclosing of the foreign substance by hemocytes [6, 9, 28]. Encapsulation is generally accompanied by melanin formation around the foreign substances. Precisely how the melanin formation occurs on the surface of a foreign substance during the encapsulation process has not been elucidated so far. We found hyper-spread cells (HSCs) in *Mythimna separata* larvae that formed a melanin deposit around themselves. A HSC attaches to the surface of a foreign substance during the early stages of encapsulation and deposits melanin in its peripheral area in a process involving phenoloxidase. This suggests that the HSCs serve as signal markers on the foreign substances to other hemocytes during the encapsulation of large foreign substances [29].

Depending on their nutritional strategy [30, 31], some koinobiont endoparasitoids allow the lepidopteran host larva to remain in the normal intake state; this allows the endoparasitoid to obtain more nutrients after parasitization. Meanwhile, they must avoid the host immune response, such as encapsulation, and obtain nourishment from the host with minimal host damage during the egg and larval stages. Almost all endoparasitoids contain the polydnavirus (PDV), which induces the host to create suitable conditions for their eggs and larvae during their development [32, 33]. The function of the PDV has been reviewed in a recent study [34]. However, a few species of braconids have a different type of particle known as the virus-like particle (VLP), which also regulates the physiological condition of the host [35]. The function of the VLP as a host regulatory factor other than the PDV is not clear yet, although their functions seem to be similar. However, it is important to point out that the PDV is a virus, which infects the host and causes the expression of the PDV gene in the host tissues or cells such as the hemocytes or the fat body; the infection ultimately results in a disturbance of the host gene expression [33, 36-42]. In contrast, the VLP is a proteinaceous particle without any nucleic acid. It is not clear whether this particle also affects the host continuously during the parasitoid larval stages.

Meteorus pulchricornis (Wesmael) (abbreviated as Mp; Hymenoptera, Braconidae) is a solitary endoparasitoid that can parasitize a wide range of lepidopteran larvae [43]. It can successfully parasitize the host *M. separata* by controlling its immune response using the *M. pulchricornis* virus-like particle (MpVLP). At the time of parasitization, MpVLP produced in the venom glands is injected along with the wasp egg into the host hemocoel, inducing apoptosis, characterized by blebbing on the cell surface of hemocytes, nuclear condensation, and ladder fragmentation with increasing caspase activity [44, 45]. In host hemocytes, especially in granulocytes, apoptosis occurs at a high frequency under *in vitro* assay conditions and decreases the population density of granulocytes after parasitization or an artificial injection of MpVLP [46, 47]. Granulocytes have very important functions in the hemocyte response, such as the encapsulation of invading substances bigger than the hemocytes. MpVLP inhibits the focal adhesion complex (FAC) on the filopodia of cells that works as a scaffold when the hemocyte extends and adheres to the surface of a foreign substance; this disturbance of FAC formation induces apoptosis [45]. As a result, MpVLP decreases the number of granulocytes of the host.

At the time of egg hatching, braconid endoparasitoids release teratocytes originating from serosal cells into the host hemocoel [48, 49]. Teratocytes seem to release physiologically active substances that inhibit the host's encapsulation response during the parasite's larval stage (Table 1) [50-52]. Although MpVLP has been shown to inhibit the extension of hemocytes in braconid endoparasitoids [46], it is unclear how the host hemocytes were regulated by teratocytes during the parasitoid larval stages, because the VLP is a proteinaceous particle and does not induce the host cells to change their gene expression, unlike the PDV. Knowledge of the physiological functions of teratocytes is fragmentary (Table 1). Teratocytes have been reported in a few studies to secrete substances that affect the host immune system. *Meteorus pulchricornis* releases

Table 1. Researches on the function of teratocytes.

Parasitoid species	Function of Tc	Identified protein	References
Meteorus gyrator	Teratocytes inhibited PO activity and released at least two kinds of protein	50, 55 kDa on SDS-PAGE	[50]
Meteorus pulchricornis	Mp19 and 18 inhibited the adhesion of hemocytes (including HSC) on the foreign surface, in both of VLP and teratocytes	Mp18, 19	this study
Microplitis croceipes	Delay the host pupation, deficient pupation		[79]
	Teratocyte injection decrease JHE-release from FB, Depress the transcription level of JHE and arylphorin		[80, 81]
	SDS-PAGE analysis: 82 kDa of riboflavin-binding protein, 74/76 kDa monomer strage protein and others related with fatbody proliferation reduced in parasitized and TSP injected larva	Teratocyte-secreted protein (TSP)	[61]
	8-20 kDa TSP affect the protein synthesis in FB	TSP (8-20 kDa)	[62]
	Teratocyte co-cultured with parasitoid larvae secret TSP14	TSP14 (14 kDa)	[63]
	Inhibition of protein synthesis related with growth of the host	TSP14	[64, 82]
Microplitis demolitor	Ploidy level of teratocyte, alteration of growth with poldnavirus plus venom		[83]
	Calyx fluid suppress the JH metabolism, but coinjection of teratocytes with calyx fluid plus venom contributes to the host developmental delay.		[65]
	Calyx fluid plus venom and teratocytes did not contribute to the elevation of JH titer		[66]
	Transcriptome analysis shows 72 teratocyte secretory proteins. The venom glands and teratocytes secrete large amount of a small number of products, which exhibit almost no overlap with one another and have functionally partitioned role in parasitized hosts, and teratocytes express high level of hymenoptaecin as antimicrobial peptides.	72 TSPs, hymenoptaecin	[34, 60]
Microplits rufiventris	Ingestion of teratocyte by parasitoid larva		[67]
	Teratocyte size became smaller by treatment with anti juvenile hormone analog; teratocytes have protective function through sequestering abnormal material		[84, 85]
Dinocampus (=Perilitus) coccinellae	Strage protein, Ingestion of teratocytes	540 kDa	[68, 69]
	Teratocyte-specific carboxylesterase	89 kDa	[86]
Toxoneuron (=Cardiochiles) nigriceps	Teratocyte injection has effects on the host development, according to the number of teratocytes injected, and on the hemolymph protein titer.		[87]

Table 1 continued..

	Inactivation of the 20HE and inhibition of secretion from prothoratic gland; teratocytes play a role to change the 20HE to polar metabolites.		[88]
	Larvae and teratocytes release the different kinds of proteins of different ages into the culture media.	Many protein bands on native and SDS-PAGE	[89]
	Parasitism-specific proteins PSP3 (56 kDa) produced from teratocytes	PSP1-116 kDa, PSP2-114 kDa, PSP3-56 kDa putative chitinase	[71, 72]
Aphidius ervi	Two proteins molecular mass 15 kD and 45 kD are abundantly secreted by 5d-teratocytes in the incubation medium, and their amino acid composition closely resemble to (that of the protein produced by teratocytes of *D. coccinellae* with nutritional role; teratocytes assist for physiological redirection and nutritional exploitation from host	p15 and p45	[73, 90]
	Teratocytes release the 15.8 kDa to bind the fatty acids	Fatty acid-binding protein Ae-FABP	[74]
	Teratocytes release enolase to mediate the host tissue digestion on the cell surface	Enolase Ae-ENO (45kDa)	[52]
	Fatty acid-binding protein (FABP) teratocytes released play a role in the nutritional exploitation from the host	FABP	[75]
Leiophron uniforms, Peristenus stygicus	Teratocytes of two species were compared: Fatty acid profiles were similar, 12 out of 22 amino acids in both species significantly differed in concentration between the 2 species.		[91]
Telenomus heliothidis	Responsible for host decomposition		[92]
Cotesia congrgata	Release the protein into the host hemocoel	More than 30 polypeptides synthesized and secreted	[93]
Cotesia kariyai	Elongate the larval period of the host with PDV plus venom		[94]
	Ploidy level increased 4-fold from 4-7d after parasitization. Many proteins were produced. Inhibition of PO activity of the host during late stage of parasitism		[95, 96]
	Extra cellular matrix decomposed after attaching on the FB	Metaroproteinase, collagenase,	[70]
Cotesia vestalis (=plutellae)	Larvicidal effect	Specific protein	[51]
	TSP has a role in altering endocrine signals	TPS	[97]
	Transcriptome analysis shows TSVP-8, -42 inhibits melanization	Teratocyte Secreted Venom Protein-8, -42 and anti-microbial peptides CvT-def 1 and -def 3.	[76]
Cotesia (=Apanteles) glomerata	Teratocyte release fungistatic material		[77]

teratocytes [47], which regulate the physiological condition of the host during the parasitoid larval stage.

In the present study, we clarified the relationship between *M. separata* HSCs, closely involved in encapsulation, and an immunosuppressive protein existing in both MpVLP and teratocytes.

MATERIALS AND METHODS

Insect rearing

Mythimna separata (Walker) (further abbreviated as Mys; Noctuidae: Lepidoptera) was selected as the host and was reared on an artificial diet (Insecta-LF®, Nihon Nohsan Co. Ltd., Kanagawa, Japan) under a long-day photo-regime (16 h light:8 h dark) at 25 ± 1 °C [47]. Adult moths were fed with a 2% sugar solution and the eggs oviposited in the narrow space of the folding papers were collected and kept in a plastic cup until hatching.

Specimens of a parthenogenetic strain of the solitary larval endoparasitoid *M. pulchricornis* were obtained in September 2004 from the common cutworm *Spodoptera litura* (Fabricius) (Noctuidae: Lepidoptera) collected in a cabbage field in Nagakute, Aichi Prefecture, Japan. The endoparasitoid uses venom and MpVLP to regulate the physiology of its lepidopteran host larvae. The venom and MpVLP, which lack nucleic acids or viral genomes, are produced by the venom gland filament and injected into the host along with wasp eggs [45]. The *M. pulchricornis* population (Nagakute strain) has been maintained with the 4th instar *M. separata* in our laboratory from 2004 to 2016. Parasitized host larvae were reared with an artificial diet in the same way as the unparasitized hosts.

Collection of Mp teratocytes

Meteorus pulchricornis develops and releases teratocytes (MpTc) into the hemocoel of the host at the time of hatching, 3.5 days after parasitization [47]. Each of the 12 parasitized hosts 4-8 d after parasitization was anesthetized with CO_2, surface-sterilized with 70% alcohol, and washed with sterilized water. Each surface-sterilized host was then dissected in a 9 cm sterilized Petri dish filled with a suitable amount of phenylthiourea-phosphate buffered saline (PTU-PBS) (PBS; pH 7.4; 8.1 mM $Na_2HPO_4 \cdot 12H_2O$, 137 mM NaCl, 2.68 mM KCl, and 1.47 mM KH_2PO_4 containing 8% saturated phenylthiourea), thereby separating MpTc out from the host hemocoel. After removing the parasitoid larvae, the solution in the Petri dish was retained for a while to remove the host hemocytes by adhesion to the base of the Petri dish. The teratocytes were made to float by lightly tapping on the edge of the Petri dish, collected with a glass pipette, and transferred to a new dish. This step was repeated twice, and the MpTc solution was then centrifuged at 800 g for 10 min at 4 °C with PTU-PBS.

Incubation of teratocytes in SF900III culture medium

To clarify the function of the proteins secreted from teratocytes, the teratocytes collected from 12 parasitized host larvae were suspended for 24 h in 500 µL of SF900III culture medium containing 8% saturated PTU and antibiotic substances (200 unit/µL penicillin and 0.1 mg/mL streptomycin). The incubation medium was used for protein analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting, and for clarifying the effect on the host hemocyte adhesion. Ten-percent SDS-PAGE was conducted after measuring the protein contents using a protein assay dye reagent (BioRad, USA) with a standard curve analysis using bovine serum albumin (BSA).

Collection of MpVLP

Venom reservoirs collected from 10 to 15 females of day 4 *M. pulchricornis* were broken into small pieces with fine forceps in 200 µL PBS and centrifuged twice at 2000 rpm for 10 min at 4 °C to remove the cell debris, and then the combined supernatant was centrifuged at 15,000 rpm for 10 min at 4 °C. The MpVLP precipitate was suspended in 1 µL PBS per female wasp.

SDS-PAGE and immunoblotting analysis

The Mp19 protein band in MpVLP [45] separated using 16 wells of 10% SDS-PAGE was cut out after visualization by SimplyBlue SafeStain (Invitrogen, Carlsbad, CA), then homogenized using two 1 ml syringes connected with a three-way stopcock (TERMO, Tokyo) and injected 4 times at 10-day intervals into the peritoneal cavity of a male mouse of BALB/C strain for producing anti-Mp19 antibody. Blood serum after decomplementation for 30 min at 56 ºC was stored separately in small aliquots at

-80 ºC until use for western blotting or immunohistrical observation to avoid lowering of the antibody titer by freeze thaw. The separated proteins with 10% SDS-PAGE were also electroblotted onto polyvinylidene fluoride (PVDF) membrane with Trans-Blot® Turbo (BioRad) to confirm the presence of Mp19 in the proteins released from each stage of teratocytes and in the VLP protein bands. The membrane was blocked for 1 h with 5% (w/v) dry skim milk (Snow Brand, Japan) in Tris-buffered saline (TBS; 20 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 0.1% (v/v) Tween-20 (TBST) and then incubated with mouse antibodies raised against Mp18 or 19, diluted to 1:2000 in a working solution (TBST-milk). Incubation with the antibodies was carried out overnight at 4 °C. The membrane was then washed 3 times with TBST-milk and incubated for 2 h at 25 ± 2 ºC with the secondary antibody HRP (Horseradish peroxidase) conjugated to goat anti-mouse IgG (Roche, Germany), and diluted to 1:2000 in TBST-milk. After washing 3 times with PBS, the membranes were detected by Light-capture (ATTO, Tokyo, Japan).

For the protein profile analysis of MpVLP by SDS-PAGE, the precipitate suspended in PBS was disrupted with an ultrasonic disruptor (TOMY, Tokyo, Japan) and separated by 10% SDS-PAGE containing 8% urea; the gel was then stained with SimplyBlue SafeStain (Invitrogen).

Analysis of amino acid sequence in N-terminus of proteins

Proteins fractionated by 10% SDS-PAGE were transferred to PVDF membrane (Immobilon P, Millipore) at constant voltage (12V) for 2 h. Semi-dry tras-blotting was performed according to the manufacturer's protocol (ATTO, Tokyo). Anode buffer I consisted of 0.3 M Tris-HCl (pH 10.4) containing 10% methanol, and Anode buffer II consisted of 25 mM Tris-HCl (pH 10.4) with 10% methanol and is used as a semi-dry transfer buffer. Cathode buffer was 25 mM Tris base and 40 mM 6-amino-n-capronic acid (pH 9.4) containing 10% methanol. The PVDF membrane after transferring was stained with Coomassie Brilliant Blue R250 staining solution (Sigma-Aldrich), and each band (18 kDa, 19 KDa) was excised from the membrane, washed with 50% methanol, and then dried. Analysis of N-terminus in each protein was performed by Amino Acid Sequencer (Applied Biosystems) using the Edoman method.

RNA extract and cDNA synthesis

Teratocytes were collected for 4-8 d after parasitization. The total RNA of the teratocytes and the venom gland from *M. pulchricornis* was extracted using an RNA extraction kit (Invitrogen) according to the manufacturer's protocol. cDNA was synthesized using 1 µg total RNA with the PrimeScript RT reagent Kit (TaKaRa Bio Inc., Shiga, Japan) at 42 °C for 60 min, digested for 30 min at 37 °C with RNase A (Sigma), and stored at -20 °C until use.

PCR and qRT-PCR

To confirm the expression of the Mp18 and 19 genes in teratocytes, polymerase chain reaction (PCR) was performed on cDNA reverse-transcribed from RNA in each stage of teratocytes using a specified primer (F: 5′-TAGTAATTCTACTGG GGTTGAC-3′ and R: 5′-TAGTCACTAGAAT CGAGGCTAT-3′) according to the DNA sequence of Mp19 (GenBank accession number AB701649.1) using AmpliTaq Gold and the following thermal cycling conditions: heating at 95 °C for 0.5 min, annealing at 52 °C for 0.5 min, and extension at 72 °C for 1 min, with a 30-cycle repetition followed by a final extension at 72 ºC for 5 min.

Each quantitative reverse transcription polymerase chain reaction (qRT-PCR) mixture (12.5 µL) contained 0.5 µL first-strand cDNA. Real-time detection and analyses were performed based on the SYBR green dye chemistry, using the SYBR Premix Ex Taq Perfect Real Time Kit (TaKaRa) and a real-time thermal cycler (Model TP-800, TaKaRa). The following thermal cycling conditions were used: 95 °C for 10 s and 40 cycles of 95 °C for 5 s and 60 °C for 30 s; this was followed by the dissociation analysis at 95 °C for 15 s, 60 °C for 30 s, and a shallow thermal ramp at 95 °C. Relative quantification of each mRNA was performed based on the threshold cycle numbers determined by the second derivatives for the primary amplification curves. The values obtained for each mRNA were normalized by the L32 mRNA amount. The primers used for qRT-PCR were 04_A07 and 03_A09, and the L32 cDNA sequence.

Hemocyte sample preparation

To clarify how Mp19 acts in the host hemocytes, hemocyte monolayer samples were prepared on a glass slide with a square boundary (approximately 24 × 24 mm) drawn using a liquid Blocker (Daido Sangyo Co. Ltd., Tokyo, Japan). Fifth instar *M. separata* larvae were anesthetized with CO_2, washed in 70% ethanol and then in sterile water, and placed on ice. The hemolymph of a d1L5 host larva was bled onto Parafilm (American Can Co., USA) on ice with a 24G needle each time after injection. To observe the effect of MpVLP on host hemocytes, d1L5 host larvae were injected with one-sixth female equivalent (solution of MpVLP collected from one female was diluted one-sixth with saline solution). The host hemolymph (10 µL) was seeded into 100 µL of SF900III medium (GIBCO) containing 8% saturated PTU (SF900III-PTU) in each well (approximately 24 × 24 mm) partitioned with liquid Blocker (Cosmo Bio, Tokyo) on the glass slide and left for 60 min. The fixed hemocyte monolayer was rinsed twice with PBS and fixed in 4% (w/v) paraformaldehyde for 20 min. After rinsing twice with TBS, the hemocyte samples were blocked with 3% (w/v) BSA in TBST for 30 min, incubated with antibody overnight at 4 °C, and washed 3 times for 15 min with TBST. The cells were then incubated for 30 min with Alexa 480-conjugated rabbit anti-mouse IgG (Invitrogen) diluted to 1:2000 in TBST. After several washes with TBS, the glass slide was mounted with 50% glycerol-PBS for observation using fluorescence microscopy (Olympus, BX-41, Japan). As negative controls, hemocytes were incubated with normal mouse serum (Sigma) (diluted to 5 µg/mL in blocking buffer) instead of the primary antibody. For observation of the cytoskeleton, the hemocyte monolayer was overlaid with PBS containing fluorescein-labeled phalloidin (0.2 mg/mL; Biotium Inc. Cosmobio, Japan) for 20 min. After washing three times with PBS, the monolayer was observed under a confocal laser scanning microscope (Carl Zeiss, Model LSM5 PASCAL, Germany) or fluorescence microscopy.

Immunocytochemical detection of teratocytes in the parasitized host

Paraffin sectioning was performed for analyzing the Mp19 protein released from teratocytes in the parasitized hosts. The parasitized host was injected with 4% (w/v) paraformaldehyde (PFA; MERCK, Germany) in PBS, kept for 20-30 minutes, dissected to remove the gut, and fixed in 4% PFA overnight at 4 °C after cutting off some pieces which were then used for paraffin section analysis. The fixed samples were dehydrated with an ethanol series and embedded in paraffin. The 8 µm sections were stained with Mayer's hematoxylin and 1% eosin Y solution according to the procedure by Sano [53]; for immunocytochemical staining, the sections were washed 3 times with 3% BSA/PBST (3% BSA and 0.1% Triton X-100 in PBS) after deparaffinization, incubated with 1000-fold diluted anti-Mp19 antibody overnight, and then incubated for 30 min with Alexa 480-conjugated goat anti-mouse IgG (Invitrogen) diluted to 0.5 µL/mL with 3% BSA/PBST. After several washes with PBS, specific binding of the antibodies was visualized using fluorescence microscopy.

RESULTS

M. pulchricornis parasitization decreased the proportion of HSCs in the hemocytes of *M. separata* hosts (Fig. 1). The rate of the number of HSCs to the total hemocyte count was 7-10% in unparasitized control hosts, but decreased to approximately 2% one day after parasitization, while after 4 days no HSCs were detected. Further, to determine if the disappearance of the HSCs was due to MpVLP, an artificial injection of MpVLP to a d1L5 unparasitized host was performed. Very few HSCs were observed 6 h after injection, compared to the control HSCs discriminated clearly by using L-Dopa as a substrate of phenoloxidase for visualizing the deposits formed on the peripheral region of the HSC spread on the glass slide (Fig. 2) [29]. This result shows that the VLP inhibits the attachment and spread of the HSCs and related granulocytes on the glass slide.

Hemocyte adherence to a large foreign substance requires the scaffold like a focal adhesion complex (FAC) that is characterized by phosphorylated tyrosine formed on the surface, which can be identified using anti-phosphotyrosine (anti-PY) [45]. Many tyrosine-phosphorylated proteins catalyzed by the focal adhesion kinase (FAK) [54-57] form the FAC as the scaffold for the adhesion of hemocytes. One-hour-long incubation of hemocytes from d0L6 unparasitized hosts on the glass slide

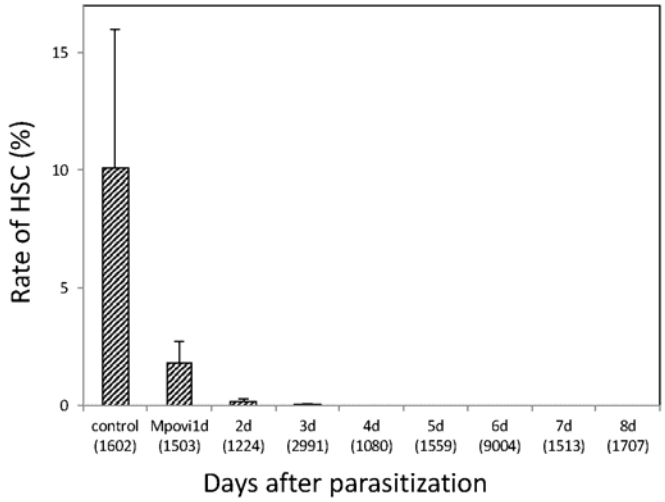

Fig. 1. Rate of hyper-spread cells (HSCs) in the host larva after parasitization with *Meteorus pulchricornis*. The number in parentheses shows the number of hemocytes counted. The HSC rate is calculated by dividing the number of HSCs by the total number of hemocytes counted.

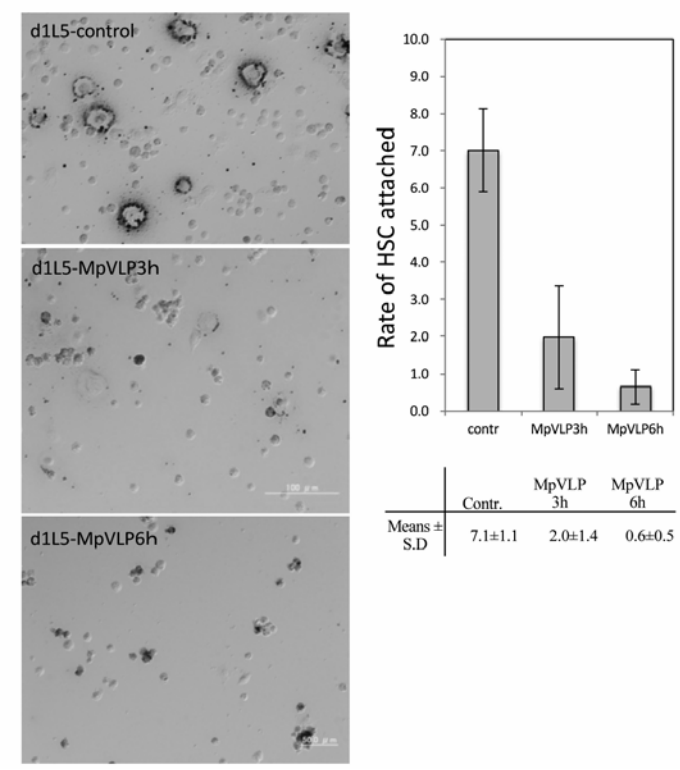

Fig. 2. *Meteorus pulchricornis* virus-like particle (MpVLP) suppresses the adhesion and/or spreading of hyper-spread cells (HSCs) on the surface of a foreign substance, such as a glass slide. Normal hemocytes retrieved from day 1 of the 5th instar (d1L5) unparasitized hosts were incubated with or without MpVLP on the glass slide of a moist chamber. The spreading of the HSCs was confirmed by melanin staining with 35% ethanol containing L-Dopa as a substrate. The right panel shows a decrease in the percentage of HSCs adhered to the glass slide when hemocytes from d1L5 host larvae were incubated for 3 and 6 h with 1/6 female equivalent of MpVLP.

resulted in the formation of FAC, shown as green circles around the hemocytes in fig. 3A, whereas the co-incubation of the hemocytes with MpVLP disrupted FAC formation, shown as the abnormal scaffold in fig. 3B.

Our previous studies revealed that MpVLP inhibits the attachment and spreading of host adhesive hemocytes *in vitro*, and eventually induces hemocyte apoptosis [44, 45]. In the present study, we focused on the rough screening of the inhibition protein from MpVLP protein profiles from SDS-PAGE with Mp19 and Mp18 protein bands (Fig. 4). These two bands were selected because of their suggestive apoptosis-inducing potential, which might match the reported characteristics of MpVLP [44]; however, the presence of this protein in MpVLP and the host hemocoel has not been confirmed.

Each Mp19 and Mp18 protein was analyzed from the N-terminus amino acid sequence by Amino Acid Sequencer using the Edoman method. The process, from the construction of cDNA to whole sequencing with other genes of *M. pulchricornis* venom, is descibed in Yokoi *et al.* [58]. MpVG04_A07 (in GenBank accession number AB701649) gene described as Mp19 in this study encoded a polypeptide of 198 amino acid residues with an estimated molecular mass of 21.6 kDa. The signal peptide analysis (SignalP4.1 [59]) for 198 amino acid residues revealed the cleavage site position between 24 and 25; the molecular mass of the active protein became 19 kDa when the signal peptide is removed during the processing of protein in the cell, corresponding to about 19 kDa protein band on the SDS-PAGE profile (Fig. 4). Also the ORF of Mp18 (referred to as MpVG03_A09 in GenBank accession number AB701655) encoded a polypeptide of 181 amino acid residues with an estimated molecular mass of 20.0 kDa, which corresponds to about 17.6 kDa protein on SDS-PAGE

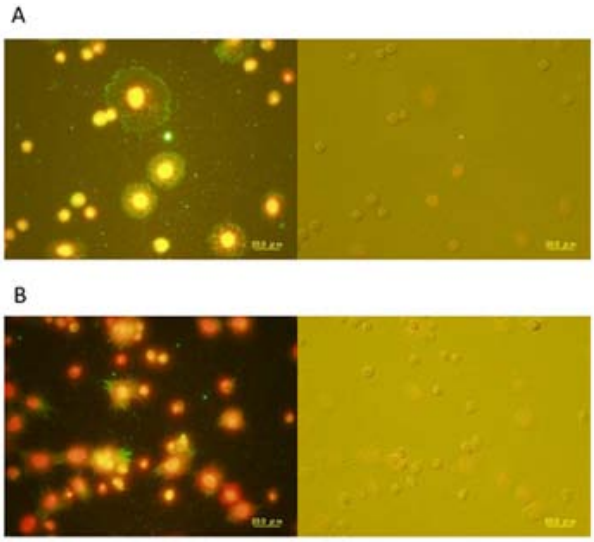

Fig. 3. A: The hemocytes from control-d0L6 of *Mythimna separata* host spreaded well on the glass slide and were stained up to the marginal edge of the hemocyte with anti-phosphotyrosine (anti-PY), which stained the tyrosine-phosphorylated proteins formed in the focal adhesion complex (FAC) as a scaffold in the peripheral region of the spreading hemocytes **B:** The host hemocytes that did not spread were stained like spine shape with anti-PY after incubation with *Meteorus pulchricornis* virus-like particle (MpVLP) for 1 h.

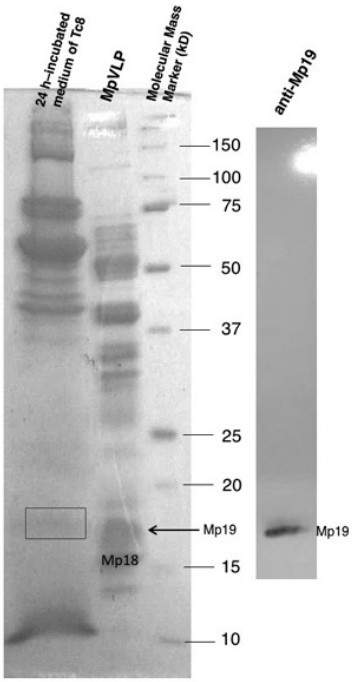

Fig. 4. Ten-percent sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) profile of both the virus-like particle (MpVLP) and the medium incubated for 24 h with 8 d teratocytes. Right panel shows immunoblotting analysis to confirm the Mp19 protein with anti-Mp19 antibody on the PVDF membrane transferred after SDS-PAGE of MpVLP proteins (Center in left panel). Pale band enclosed in a rectangle on the profile of proteins released from 8 d teratocytes seems to correspond to Mp19.

analysis when removing signal peptides from 181 amino acid residues with cleavage site position between 21 and 22. Each molecular mass on the SDS-PAGE profile represents an active form of protein after protein processing in the cells. These two proteins shared some physicochemical properties. Both had alkaline isoelectric points and had hydrophobic stretches near the N-terminal region. Finally, the SDS-PAGE analysis suggested that both were glycine- and serine-rich in the C-terminal region. This means it is possible that they are some kind of glycoprotein, which functions to inhibit the hemocyte behavior.

To confirm the function of the Mp19/18 protein, Mp19 and/or Mp18 gene silencing (RNAi) was performed in an earlier study and the results appeared in Applied Entomology and Zoology [58]. Two genes showed approximately 50% similarity to each other; because of this similarity, we focused on Mp19 in this study. Also in this study, the adhesive hemocytes from each *M. separata* host larva parasitized by knockdown wasps or wild-type wasps, namely plasmatocytes and granular cells, were observed using phalloidin staining to compare the effect on the actin fibers in the filopodia of the plasmatocytes (Fig. 5A, left and center panels) and

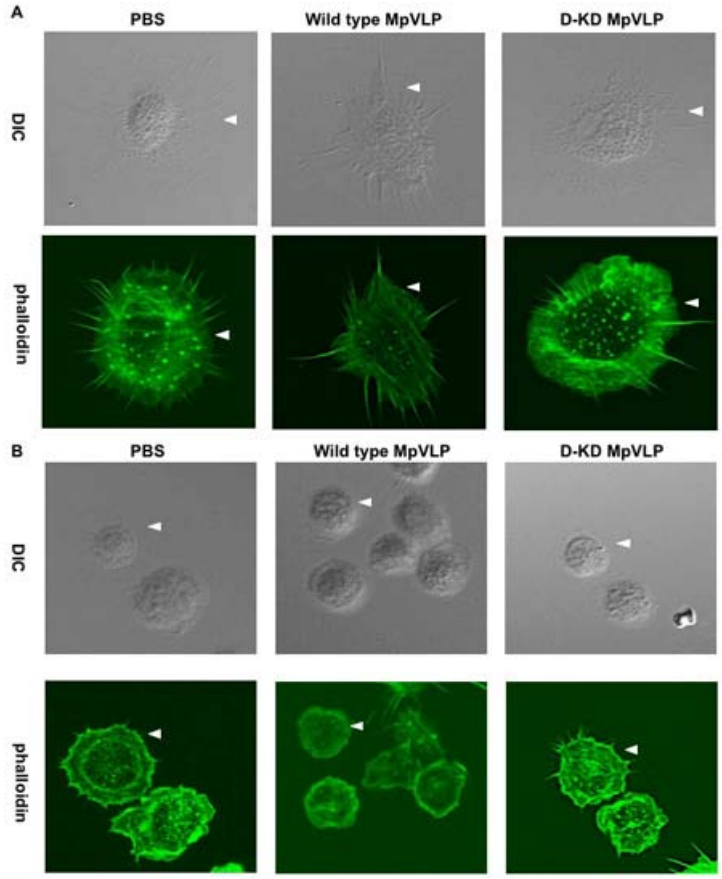

Fig. 5. The influence of *Meteorus pulchricornis* virus-like particle (MpVLP) on *Mythimna separata* adhesive hemocytes *in vitro* and the effects of knockdown of MpVLP components encoded by 04_A07 and 03_A09. MpVLP, which is prepared from either control or knocked-down wasps, was mixed with *M. separata* hemocytes and incubated for 30 min on a glass slide. After washing, fixation, and staining with fluorescein-phalloidin, adhesive hemocyte species were observed using a confocal laser scanning microscope. As a negative control, PBS was included instead of the MpVLP suspension. **A:** Plasmatocyte. **B:** Granular cells. Each category is composed of a pair of images by differential interference contrast (DIC) and fluorescent observation. Left, PBS. Center, treatment with wild-type MpVLP. Right, treatment with MpVLP from wasps pretreated with dsRNA of both 04_A07 and 03_A09 (D-KD MpVLP). Arrowheads indicate the boundaries of attached cells (edges of lamellipodia).

the granulocytes (Fig. 5B left and center panels). Hemocytes incubated with wild-type MpVLP suppressed the extension of filopodia and lamellopodia, compared to that of PBS incubation. In contrast, deficient MpVLP with double knockdown (D-KD) failed to suppress the spreading of hemocytes (Fig. 5A and B, right panel), suggesting that these two MpVLP factors had a cumulative effect on hemocyte spreading.

The antibody of Mp19 rescued the HSC spreading to 6-7% of the total hemocyte count in spite of MpVLP included in the incubation medium (Fig. 6).

To ascertain the attachment and the uptake of the Mp19 protein into the host hemocytes, the hemocytes from the host larvae at different times after injection of MpVLP were stained with anti-Mp19 antibody.

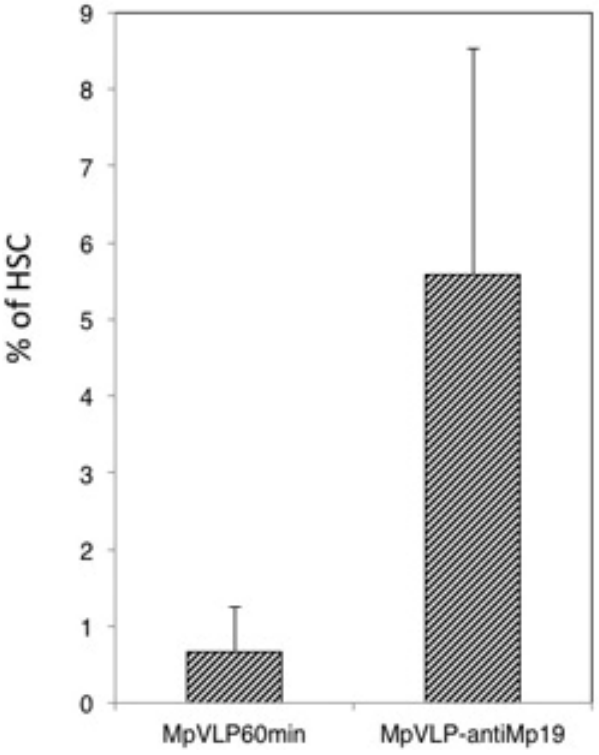

Fig. 6. The inhibitory effect of *Meteorus pulchricornis* virus-like particle (MpVLP) on the spreading and adherence of hyper-spread cells (HSCs) is counteracted by the addition of anti-Mp19 antibody. Normal hemocytes from unparasitized d1L5 host larvae were incubated for 60 min with MpVLP or MpVLP containing anti-Mp19 antibody (1 µL/100 µL SF900III incubation medium).

The cytoplasm and the granules in the hemocytes taken from the hosts 1 h after injection was stained well with anti-Mp19 antibody (Fig. 7). The number of hemocytes stained with antibody decreased gradually after 1 h post-injection. A few hemocytes was stained 6 h later and even 1 d post-injection on the granules in the cytoplasm, but no hemocyte was stained 2 d later after MpVLP injection (Fig. 7).

To clarify whether the Mp19 gene was also expressed in teratocytes, PCR using cDNA templates extracted from 5 to 8 d teratocytes was performed. It showed that the Mp19 gene was expressed in all developmental stages of teratocytes (Fig. 8). The relative amount of mRNA from MpTc4 through MpTc8 showed a peak value at MpTc5 and decreased on day 7 and day 8 (Fig. 9). On the other hand, the relative amount of mRNA from Mp larvae showed a peak value 6 days after oviposition, when just ecdysed to the 2nd instar. The Mp18 gene expression had a lower value, which was approximately one fortieth of the level of Mp19 expression.

Western blot using anti-Mp19 revealed that teratocytes at every developmental stage released approximately 65-kDa protein. In contrast, inside the teratocytes at each stage, proteins of different molecular weights (from 17.8 kDa to 65 kDa) were produced (Fig. 10).

The incubation medium of *M. pulchricornis* teratocytes in all developmental stages suppressed the spreading of HSC and granulocytes on the glass slide. The cytoskeleton of hemocytes stained by phalloidin confirmed the inhibition of the extension of actin fibers in many hemocytes appearing in round shape compared to that of control hemocytes (Fig. 11 and left panels in Fig. 12), and the host hemocytes with the cytoplasm stained by anti-Mp19 antibody increased when they were incubated in the medium after 6 d old teratocytes (MpTc6) (left panels in Fig. 12).

Further, to observe the actual existence of the Mp19 protein released from teratocytes and larvae, paraffin sections of host larvae were stained with anti-Mp19 antibody, demonstrating that the Mp19 protein (as small granules stained with FITC shown in fig. 13D, E, F) existed in multiple locations and around the tissues inside the host, such as the fat body or the teratocytes (Fig. 13). Moreover, teratocytes moved between the host tissues with morphological change (Fig. 13A, C). These results demonstrated that the

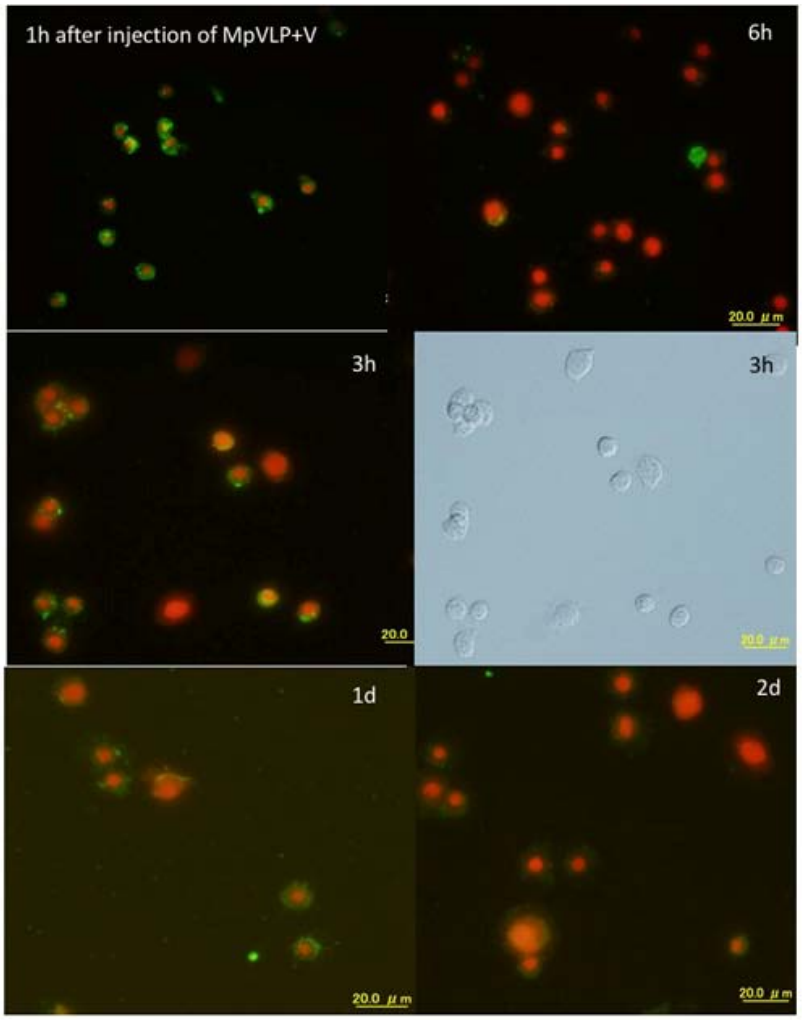

Fig. 7. Hemocytes from hosts at several times after the injection of *Meteorus pulchricornis* virus-like particle (MpVLP) and venom, where the intake of the Mp19 protein (recognized with the FITC green color) was detected using the anti-Mp19 antibody. The nuclei were stained with 1% propidium iodide.

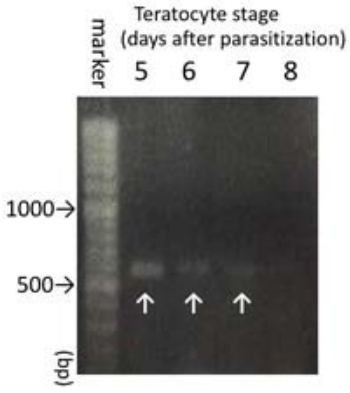

Fig. 8. The PCR profile of the Mp19 gene in teratocytes at each developmental stage.

Mp19 gene products expressed in the venom gland of adult female wasps and in the teratocytes and larvae are complementary to each other. The Mp19 protein was polymerized as a large protein molecule in the teratocytes (Fig. 10) and released into the host hemocoel to inhibit hemocyte spreading.

DISCUSSION

We summarized the information on the substances released from teratocytes that has been reported in previously published studies (Table 1), whereas Strand [60] had recently reviewed the function of teratocytes from physiological viewpoints. Several studies have described teratocyte involvement in

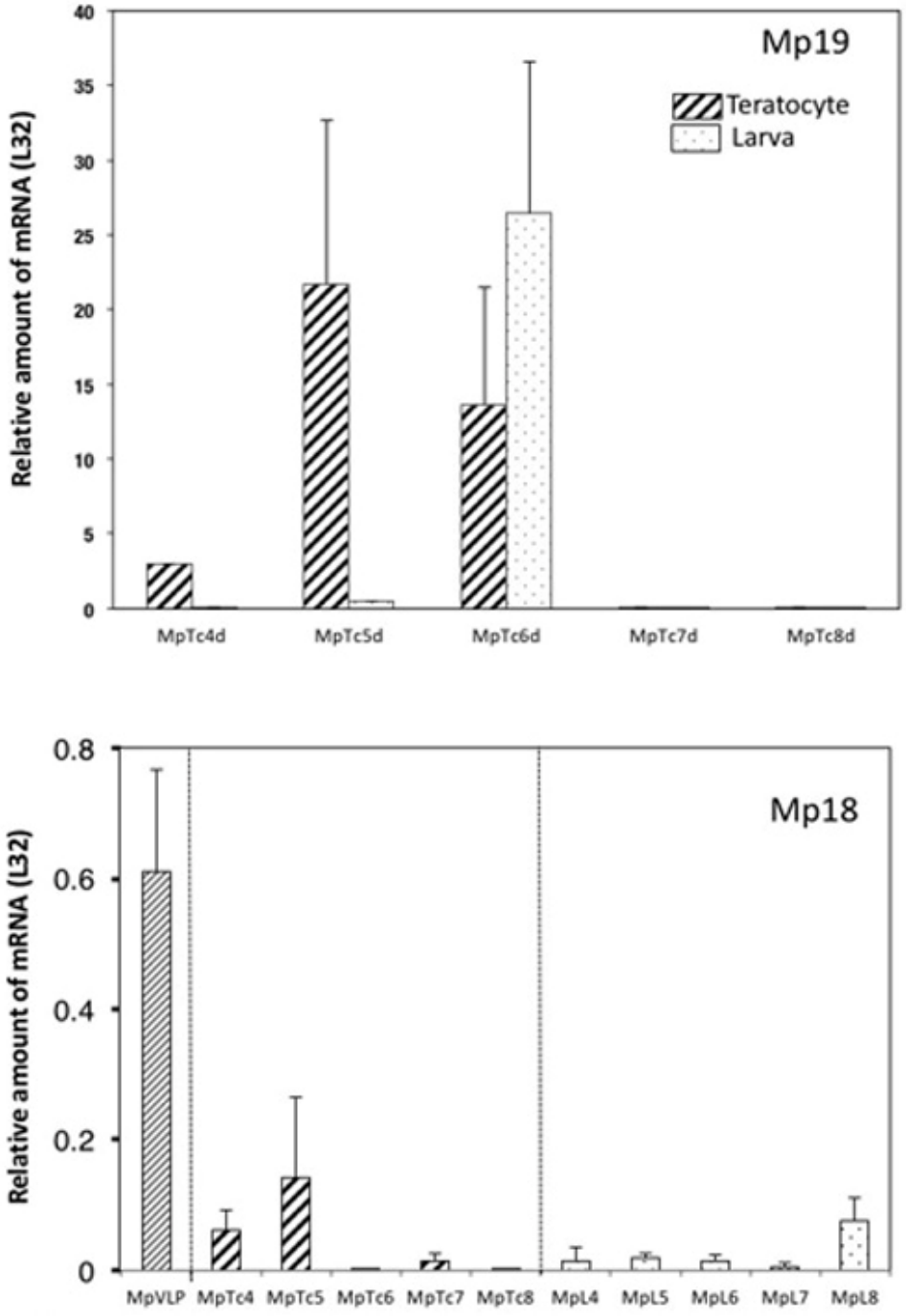

Fig. 9. The relative amount of Mp19 and Mp18 mRNA extracted from teratocytes (shaded bar) and parasitoid larvae (dotted bar) at each developmental stage. In Mp18 panel, relative amount of Mp18 mRNA expressed in Mp venom glands is shown as positive control on the left side.

the elongation of the larval period due to hormonal imbalance. As an example, TSP 14 was found to inhibit the synthesis of proteins related to the growth and development of *Microplitis croceipes* [61-64] and to affect the juvenile hormone (JH) level in *Microplitis demolitor* [65, 66]. On the other hand, teratocytes serve as food storage for the parasitoid larvae [67-69] through either direct ingestion or

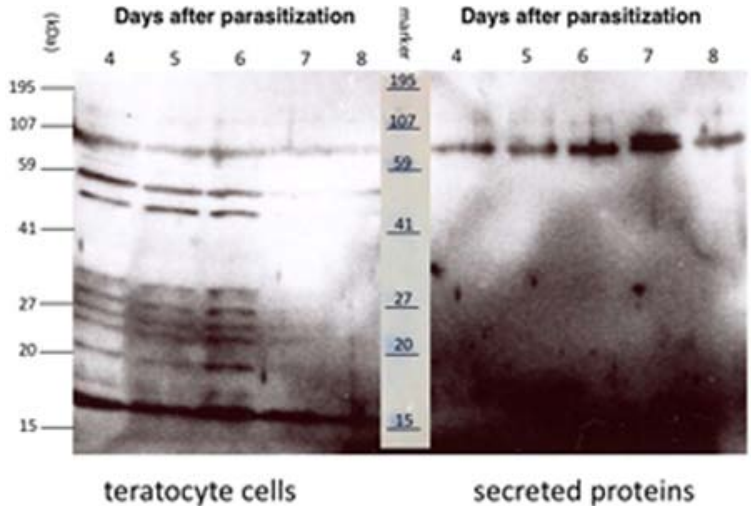

Fig. 10. Western blotting of proteins separated with 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using anti-Mp19 antibody. Protein samples on the left side were prepared from homogenized teratocytes at each post-parasitization stage, while those on the right side were prepared from culture medium (SF900III containing 8% saturated PTU, streptomycin, and penicillin) after 24 h incubation, with the protein contents adjusted to 25 µg per lane.

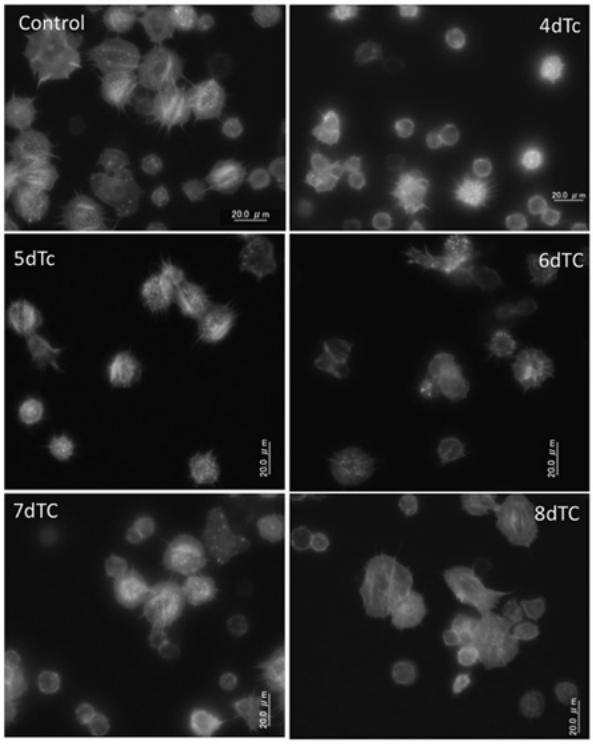

Fig. 11. Hemocytes affected by the teratocyte incubation medium at each stage, detected by phalloidin staining after a 1 h incubation with the culture medium (SF900III+8% saturated PTU solution), which had been incubated with teratocytes at each developmental stage for 24 h.

decomposition. Teratocytes act on the cellular matrix of the fat body in the host to exclude the contents, which the larvae then consume to gain sufficient nutrition for the later growth and development of the parasitoid larvae [70]. In a different example, the chitinase of *Toxoneuron* (=*Cardiochiles*) *nigriceps* [71, 72] appears to be responsible for decomposing the cellular matrix of the fat body. A reduction in fat body proliferation and an increase in the protein contents of the host's hemolymph have also been reported in *M. croceipes* [61]. In *Aphidius ervi*, the fatty acid-binding protein was shown to be related to nutritional regulation [73-75], and the enolase on the cell surface was found to be multifunctional [52]. Strand and Burke [34] and Gao *et al*. [76] determined and grouped many kinds of proteins or peptides produced in the teratocytes by conducting transcriptome analyses of teratocytes in *M. demolitor* and *Cotesia plutellae*. Teratocytes from *M. demolitor* produced 72 teratocyte secretory proteins, with no overlap with the proteins found in venom and *M. demolitor* bracovirus (MdBV). Antimicrobial peptides such as hymenoptaecin likely compensate for MdBV-mediated immunosuppression. TSVP-8 and TSVP-42 in *C. plutellae*, however, were found to inhibit the melanization related to the avoidance of the host immune function, and 2 anti-microbial peptides inhibited bacterial growth [76]. Fungistatic

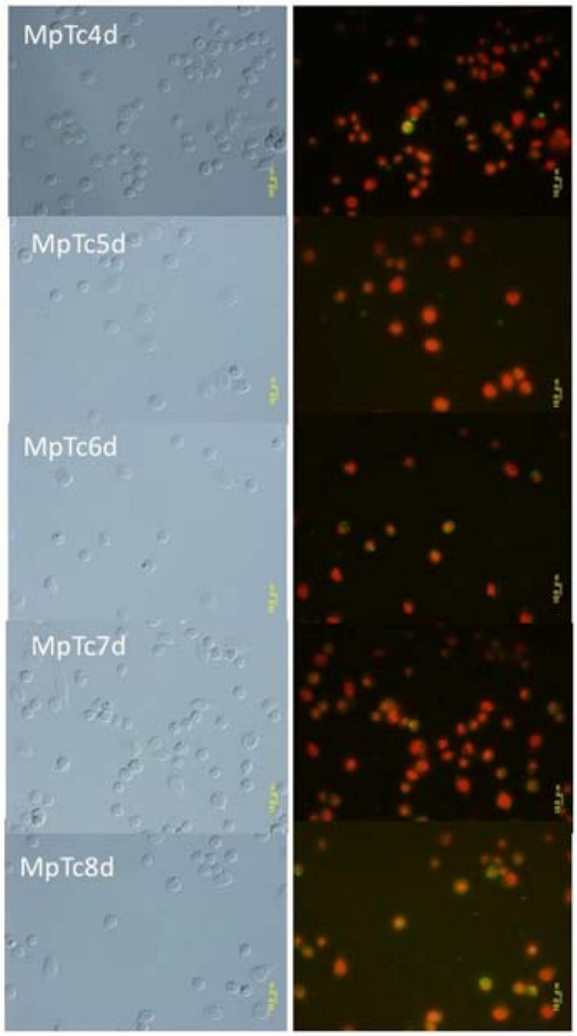

Fig. 12. Hemocytes from unparasitized hosts were incubated for 1 h with culture medium prepared by incubation for 24 h with teratocytes at each developmental stage. Mp19 antibody was used for the detection of the Mp19 protein inside hemocytes. The nuclei were stained with 1% propidium iodide.

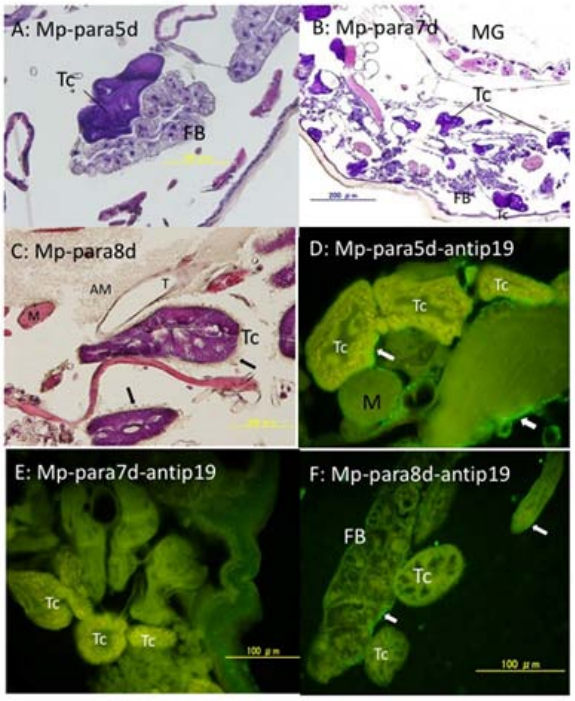

Fig. 13. Paraffin sections of hosts parasitized by *Meteorus pulchricornis* at each developmental stage. Six-micrometer-thick paraffin sections were stained with a Mayer's hematoxylin and 1% eosin solution (A, B, C) and stained with anti-Mp19 antibody (×2000 dilution to the original serum) overnight at 4 °C (D, E, F). Tc: teratocyte, FB: fat body, MG: midgut of the host larva, T: trachea of the host larva. M: Muscle. Especially in panel C, the amorphous materials (AM) like porteinous material and microvilli (as indicated by an arrow) on the peripheral edge of 8 d teratocytes (Tc) were observed. In panels D to F, small green particles stained with anti-Mp19 antibody are indicated by white arrow.

material released from teratocytes was also found in *Cotesia* (=*Apanteles*) *glomerata* [77].

We focused on the disappearance of the HSCs after parasitization, because HSCs adhere to, and spread on, the foreign surface by actin polymerization and FAC formation, which results in melanin deposition around the HSCs, which in turn assists and accelerates the adhesion of other hemocytes [29]. Both Mp-parasitization and MpVLP suppress the spreading of filopodia and lamellipodia of the host hemocytes and induce apoptosis, decreasing the number of granulocytes of the host larva [44, 46]. Incubation with MpVLPs from parasitoid wasps with double knockdown of the MpVG03_A09 and MpVG04_A07 (Mp18 and Mp19 genes) [see 58] resulted in poor filopodia of granulocytes when compared to those of hemocytes from the hosts injected with PBS. Furthermore, the coexistence of anti-Mp19 antibody under incubation of hemocytes with MpVLP was rescued to elevate the proportion of HSCs to that in the unparasitized state (Fig. 6). These results showed that Mp18 and Mp19 might be related to disruption of the hemocyte extension while the cell adhered on the foreign substance.

Although almost all adhesive hemocytes were observed to take MpVLP into the cells within 1 h

after artificial injection, the number of cells stained with the antibody decreased with time, showing that the hemocytes affected by the function of MpVLP were eliminated, and hemocyte with little influence by MpVLP proteins remained. This result was supported by previous studies [45, 46], which reported high activity of caspase 3-like proteinase 3 h after MpVLP treatment and a decrease in granulocytes with time after parasitization. Subsequently, when hemocytes were incubated with the culture medium of teratocytes in the developmental stage, especially in the culture medium with day 5 to day 8 teratocytes, hemocytes affected by the Mp19 protein increased in number. This appears to be related to the expression of the Mp19 gene reaching its maximum levels in 5 d teratocytes and 6-day-old parasitoid larvae. Another interesting observation was that although teratocytes were always round shaped when taken out of the host hemocoel, they actually moved through the space between the host tissues. Even large granules released from teratocytes might be delivered to the surface of the host tissue, such as fat body, supporting the results of the western blot and paraffin section analyses.

Protein packed into Mp-virus like particle (VLP) in the wasp venom glands [44] was injected with an egg into the host hemocoel which affect the host hemocyte behavior by inhibiting the adhesion of hemocytes; specifically malfunction of adhesion might induce apoptosis in the deduced HSCs through the suppression of actin polymerization and FAC formation. Suzuki and Tanaka [44] reported that many hemocytes existed in the parasitized host, although the hemocyte decreased in number. This suggests that the parasitoid eggs and larvae can avoid encapsulation through inducing the failure of FAC formation or actin polymerization by the Mp19 protein mainly, which is found in the MpVLP and is released from teratocytes. As a result they cause apoptosis or inhibition of the adhesion on some kind of hemocytes such as HSC.

CONCLUSION

Braconid parasitoids produce the PDV (BV) in the calyx region of the ovary and inject it into the host hemocoel with the egg(s), which additionally generate teratocytes from the serosal membrane in the host hemocoel [60, 78]. PDV injected into the host is a virus and continuously produces regulatory substances to protect the parasitoid eggs and larvae by inducing a malfunction of the host's defense system. MpVLP in *M. pulchricornis* is a proteinaceous particle, and thus different from a virus, although it exhibits some structural similarity to a virus in that the proteins are packed as a capsule. Its role of protecting the parasitoid larva appears to be transient [44], because the hematopoietic organ is not damaged by MpVLP attack [46]. The effect of immune suppression must therefore be continued by the teratocytes or the larva. In the parasitoid larval stage, teratocytes and larvae were found to release Mp18 and Mp19, which were included in the protein package of the VLP and suppressed the adhesion and extension of the hemocytes, especially HSCs, as one of the species of granulocytes. Further research with a transcriptome analysis is required to elucidate the relationship between the proteins secreted from teratocytes and hemocyte regulation.

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CONFLICT OF INTEREST STATEMENT

There are no ethical considerations or conflicts of interest with regard to this manuscript.

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