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RESEARCH ARTICLE

A Novel Site for Hematopoietic Organ in *Bombyx mori* L. (Lepidoptera: Bombycidae)

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Summary

A novel site for hematopoietic organ closely associated with the dorsal vessel (HPO-DV) in the fifth-final larvae of *Bombyx mori* L. was described. HPO-DV is composed of several lobes located very close to the dorsal vessel in the thorax and in the abdominal segments of the larvae. The anterior lobes contain big secretory cells. Loose hemocyte clusters among the secretory cells were observed particularly in the posterior lobes. When the HPO-DV was cultured in the absence of hemolymph; prohemocytes, plasmatocytes, and granulocytes were released from the hematopoietic organ. Cultured hemocytes from two sources, released from HPO-DV and circulating in the hemolymph, were compared under inverted microscope. They remained the suspended in culture medium. Conversely, the cells in the hemolymph immediately adhered on the flask. This different behavior of the cells obtained from different sources implies that the cells produced from the HPO-DV probably continue to differentiate in the hemocoel.

Keywords: *Hematopoietic Organ, Cell and organ cultures, Hemocytes, Bombyx mori*

Bombyx mori L. (Lepidoptera:Bombycidae)'de Yeni Hematopoietik Organ

Özet

Bombyx mori L.'nin son evresinde dorsal damar ilişkili yeni hematopoietik organ (HPO-DD) belirlendi. HPO-DD larvanın toraks ve abdomen segmentlerinde dorsal damarın iki yanında, onunla yakın ilişkili yer alan ve loplardan oluşan bir organdır. Anterior loblar büyük salgı hücrelerini içermektedir. Özellikle posterior loblarda gruplar halindeki hemositler salgı loplarının arasında yer aldığı gözlendi. Doku kültürü ortamında, organdan prohemosit, plasmatosit ve granulosit hücre tipleri salınmıştır. HPO-DD'dan ve dolaşımdaki hemositlerin hücre kültürleri inverted mikroskop altında karşılaştırıldı. Bu hücreler kültür ortamında zemine tutunmayıp ortamda asılı bir şekilde kaldı. Diğer taraftan hemolenfteki hemositler kültür kaplarına aktarıldıktan sonra hemen zemine tutunduğu gözlendi. Bu davranış farklılığı HPO-DD'dan salınan küçük hemositlerin hemosölde farklılaşmaya devam ederek dolaşıma katıldıklarını düşündürmektedir.

Anahtar sözcükler: *Hematopoieitik organ, Hücre ve organ kültürleri, Hemositler, Bombyx mori*

INTRODUCTION

The sites of the hematopoietic organs (HPOs) producing the hemocytes and their structural complexity vary with the insect's phylogenetic position [1,2]. HPOs in Diptera are generally connected to both sides of the dorsal vessel [2]. In *Drosophila,* 4-6 pairs of lobes are associated with the dorsal vessel in the thorax [2-8]. On the other hand, in *Calliphora,* 5-8 islets of dividing cells are found in abdominal segments [2]. In Lepidoptera species; *Bombyx mori* [9-11], *Manduca sexta* [12,13], *Pseudoplusia includens* and *Spodoptera frugiperda* of HPOs are attached to imaginal wing discs [14]. The most highly organized organ is found in the housecricket, *Gryllus,* a member of the order Orthoptera [2]. In general, HPOs in insects with the exception of lepidopteran species are

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located near the dorsal vessel in the thorax and the abdominal segments.

The lepidopteran hematopoietic process is poorly understood [10] and questions concerning hemocyte proliferation, differentiation and mobilization still remain unsolved [9,10,15]. Little is known about the development or the transformation of the different hemocyte types into one another in the silkworm, *B. mori* [11]. The physiological properties of hematopoiesis in cultured hematopoietic organs have not been studied in detail. Therefore, *in vitro* tissue culture of the hematopoietic organ (HPO) can be a useful tool for the study of insect hematopoiesis [10] and reveal much information about the differentiation of hemocytes [16].

In this paper, a novel site for HPO, in additon to HPOs attached to imaginal wing discs [9,12,14], was described that lies adjacent to the dorsal vessel in *B. mori*. The hemocytes are discharged from the lobes of HPO into the medium, and it has been confirmed by tissue culture that this organ is an HPO. Finally, this study is the first demonstration of the presence of an HPO closely associated with dorsal vessel in *B. mori* that had not previously been described in the silkworm larvae.

MATERIAL and METHODS

The Silkworm and Its Hemocytes

The larvae of silkworm, *Bombyx mori* were cultured on the leaves of the mulberry at 24±1°C, 70% relative humidity, and under a photoperiod of 12:12 (light and dark) [17,18]. In this study, we used the fifth or last instar larvae of silkworm, designated in text as L5. The prohemocytes, plasmatocytes and granulocytes, being effector cells to the immune response and hematopoiesis [2,19,20], comprised approximately 85-95% of all hemocytes in the last instar larvae of Lepidoptera [12,13,16].

Silkworm hemocytes were classified into three morphotypes using the criteria described by Lackie [2], Yamashita and Iwabuchi [16] and Nakahara et al.[10]: (1) the prohemocyte is a small, round non-adhesive cell and thin cytoplasm without cytoplasmic inclusions; (2) the plasmatocyte is a pleomorphic, usually oval, adhesive cell; (3) the granulocyte is a round adhesive cell with numerous small granules.

Preparation and Culture of Hematopoietic Organ and Hemocytes

Hematopoietic organs of L5 larvae were dissected under binocular microscopy (Carl Zeiss, 9901). HPOs were washed three times with 0.01 M Phosphate buffer saline (PBS) (pH:7.3) and transferred to a plastic petri dish (Falcon® 35x10 mm) with 2 ml of culture medium. These culture dishes were incubated at 26°C and natural humidity. The release of hemocytes from the HPOs was observed under an inverted microscope (Olympus CK40) and photographs were taken at 24 h intervals.

The cell culture medium was composed of Grace's Insect Medium (Sigma, G8142) with fetal calf serum (Sigma, F3018) and antibiotic-gentamycin (Gibco). In order to carry out the cell culture, 2 ml medium and 1 ml medium with hemolymph were added to each disposable plastic petri dish. The cells were maintained at 26°C and natural humidity.

Light Microscopic Structure of Hematopoietic Organ

Light microscopic investigation was performed on the semi-thin sections of Epon 812 embedded materials. For this purpose, the dissected HPOs were fixed in Karnovsky [21] fixative, pH 7.4 at 4°C, and post fixed in 1% OsO_4 in phosphate buffer [22]. After dehydration, the organ samples were embedded in Epon 812. Semi-thin sections (1 µm) stained with Toluidin Blue were used for light microscopic investigation.

RESULTS

In the investigation of HPO, a lobulated compact structure was identified along the dorsal vessel in the thorax and the abdomen segments of larvae of *Bombyx mori*. To determine whether this structure is an HPO, an *in vitro* tissue culture system was established. During the monitoring under the inverted microscope, a number of hemocytes released from the folds of the HPO were observed after 24 h *(Fig. 1)*. According to the description by Lackie [2], Yamashita and Iwabushi [16] and Nakahara et al.[10], these cells were prohemocytes, plasmatocytes and a small number of granulocytes. Granulocytes appeared round, while plasmatocytes were spindle or star-shaped. Prohemocytes were also round cell but noticeably smaller than the other types of hemocytes. In addition, the hemocytes that were discharged from the cultured HPO lobes remained suspended, and they did not adhere to the surface of the flask *(Fig 2a)*. Conversely, when hemocytes in circulation were kept in the culture environment, they immediately adhered and spread across the flask bottom to form a layer *(Fig 2b)*. These observations suggest that differentiation of hemocytes released from HPO still continues into morphotypes in the hemolymph.

We have termed the organ as a novel site of HPO associated with the dorsal vessel as Hematopoietic Organ Related Dorsal Vessel (HPO-DV). The organ is

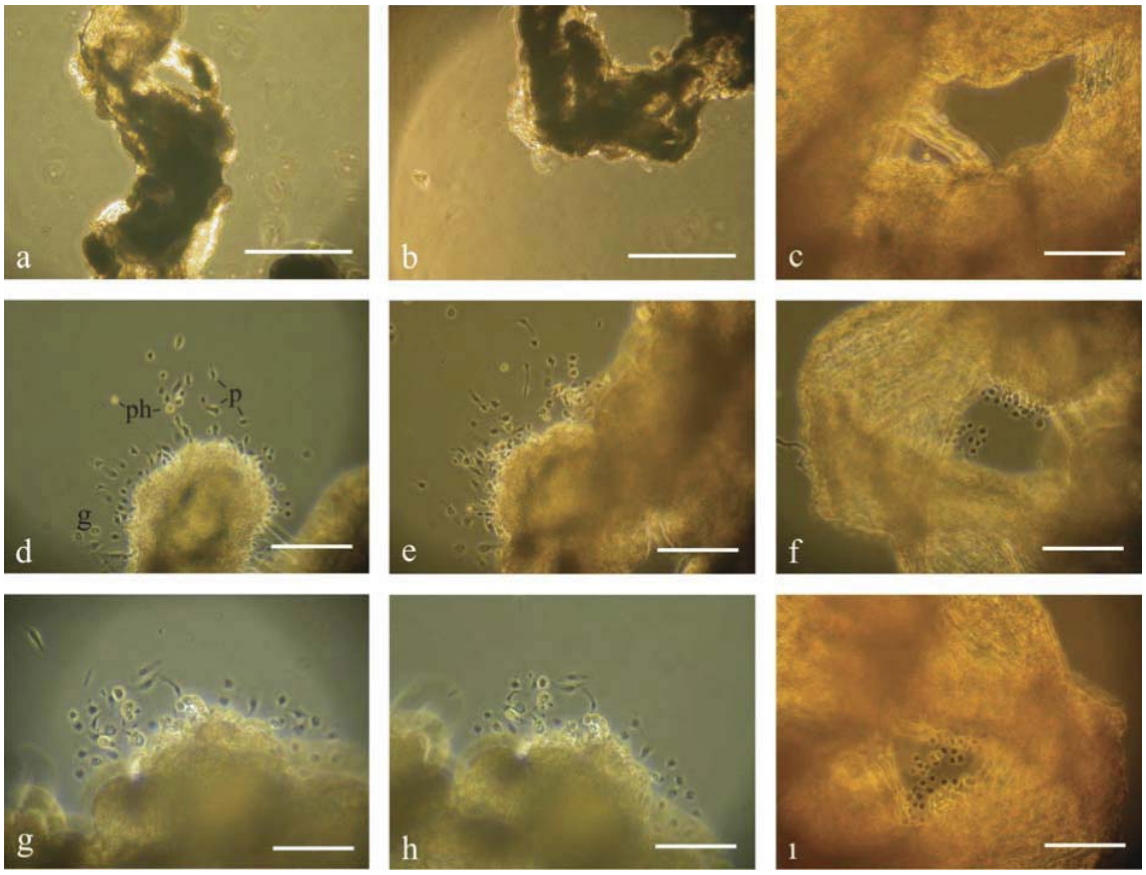

Fig 1. Cultured HPO-DV lobes from L5-larvae. **a, b, c.** No hemocyte release after the first 30 min in culture medium, **d, e, f.** Hemocyte release on the second day, **g, h, i.** Hemocyte release on the third day. **p:** plasmatocytes, **g:** granulocytes, **ph:** prohemocytes (Bar 100 µm)

Şekil 1. Kültür ortamında HPO loplarından hemositlerin salınması. **a, b, c.** Kültür ortamına koyulan HPO'nun ilk 30 dakika sonraki yapısı ve hemosit salınımı yok, **d, e, f.** İkinci gün HPO'nun loplarından salınan hemositler, **g, h, i.** Üçüncü gün loplardan salınan hemositler. **p,** plazmatositler, **g,** granulositler, **ph,** prohemositler (Bar 100 µm)

composed of several lobes *(Fig 2c)*. They are located along the surface of the dorsal vessel and maintain very close contact with it. The inner surface of the organ's lobes lies up against the dorsal vessel and outer surface faces the hemocoel. All lobes were surrounded by a continuous layer of basement membrane or stroma *(Fig 2d)*.

HPO-DV exhibits morphological differences between the anterior and the posterior lobes along the extension of the organ in the thorax and the abdominal segments of the larvae. Anterior lobes were primarily composed of big secretory cells. Also, there are some differences among the anterior lobes of HPO-DV according to the included granules. Some of lobes contain cells with small and round granules. In neighboring lobes, similar big cells were filled with secretory granules very different in appearance *(Fig 2e)*. The posterior lobes of HPO-DV include secretory cells and loose-associated different hemocyte clusters *(Fig 2f)*.

DISCUSSION

It has been suggested that the HPO of lepidopteran insects, *Bombyx mori* [9-11], *Manduca sexta* [12], *Pseudoplusia includens* and *Spodoptera frugiperda* [14] are located in imaginal wing discs. However, we have identified a novel site for hematopoietic organ in silkworm larvae. The morphology and the structure of the organ are very similar to the HPO described in *Drosophila* sp. It is suggested that in *Drosophila* larvae, anterior lobes of HPO contain especially secretory cells while the posterior lobes consist of essentially prohemocytes like in *B. mori*. These anterior lobes produce various proteins including collagen, peroxidasin and antifungal peptides following an immune challenge [3]. Secretory cells in the anterior lobes of the HPO-DV of *B. mori* can also be responsible for the synthesis of similar defense molecules for humoral and cellular immune response.

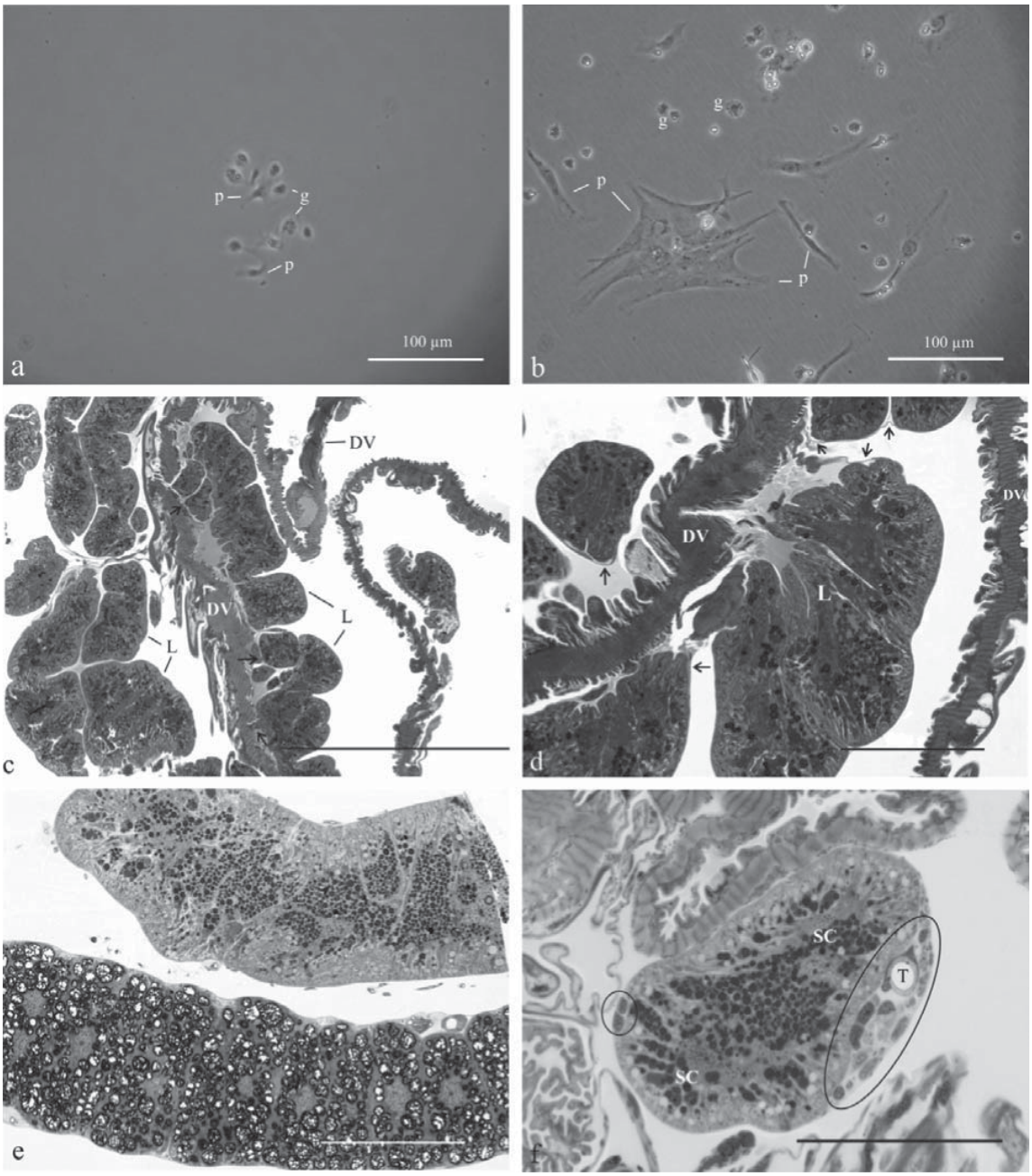

Fig 2. Hemocytes in culture medium (a, b): **a.** released from HPO-DV to culture medium, **b.** obtained from circulating hemolymph. **p.** plasmatocytes, **g.** granulocytes (Bar 100 µm). The general structure of HPO-DV composed of several lobes (c, d): **c.** close contacts (→) between the lobes of HPO-DV and the dorsal vessel (Bar 200 µm), **d.** a stromal coat (→) surrounding the lobes (Bar 50 µm). **L;** lobe, **DV;** dorsal vessel (1 µm epon section stained with Toluidin blue). **e.** anterior lobes of HPO-DV include secretory cells containing different secretory granules (1 µm epon section stained with Toluidin blue) (Bar 50 µm). **f.** posterior lobe of HPO-DV containing both secretory cells (SC) and different loose hemocyte clusters (in circles). **T;** trachea (1 µm epon section stained with Toluidin blue) (Bar 50 µm)

Şekil 2. Kültür ortamındaki hemositler (a, b): **a.** HPO-DV'dan kültür ortamına salınan hemositler, **b.** hemolenfte dolaşan hemositlerin kültürü, **p.** plazmatositler, **g.** granulositler, **ph,** prohemositler (Bar 100 µm). Loplardan oluşan HPO-DV'nin genel yapısı (c, d): **c.** dorsal damar ile HPO-DV arasındaki yakın ilişiki (→) (Bar 200 µm), **d.** lobları saran bir bazal örtü (→) (Bar 50 µm). **L;** lop, **DV;** dorsal damar (1 µm'lik epon kesit, Toluidin mavisi). **e.** farklı salgı granüllerine sahip salgı hücrelerinden oluşan HPO-DV'nin anterior lopları (1 µm'lik epon kesit, Toluidin mavisi) (Bar 50 µm). **f.** salgı hücreleri (SC) ve gevşek yapıda hemosit topluluklarını (daire içinde) içeren HPO-DV'nin posterior lobu. **T;** trake (1 µm'lik epon kesit, Toluidin mavisi) (Bar 50 µm)

Several types of circulating hemocytes were characterized in larval Lepidoptera, but there is no agreement as to where blood cells are produced and how their populations are maintained [14]. Nakahara et al. [10] and Ling et al.[11] showed that most of the cells discharged from the larval HPO near to the imaginal wing discs in *B. mori* were plasmatocytes, prohemocytes and granulocytes. They concluded that young hemocytes produced from the organ have a great ability to multiply by mitotic division in circulation. In another study, hemocytes discharged from cultured HPO of *M. sexta* either adhered and spread on the culture surface or remained suspended in the culture medium. The only adherent cells spreading to the glass substrate were plasmatocytes. The prohemocytes were non-adherent and remained in suspension [12]. The behavior of the cells derived from the HPO-DV in *B. mori* was quite similar to the observations made in earlier studies. When circulating hemocytes were added to culture dishes, they did not adhere or spread at first. The cells were later allowed to settle and adhere to the glass coverslip for a time. Consequently, we propose that hemocytes discharged from HPO-DV are likely to continue to differentiate in the hemocoel.

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