Studies of the Post Infectious Transmission of Nucleopolyhedrovirus Associated with the Circulatory System of Silkworm

~血液系を介したカイコ核多角体病ウイルスの体内伝播の研究~

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Contents

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Abstract:

Hemocytes of open circulatory system work as immune system and protect them from pathogens. Baculovirus can infect hemocytes, and the infection of hemocytes is possibly playing the important role of systemic infection of this virus. Two systemic infection routes are supposed for the post infectious transmission of Nucleopolyhedrovirus (NPV), via hemolymph and via tracheal lymph systems. Understanding the systemic mechanism of how virions spread around the whole body and how it triggers high and sudden host's mortality is one of the important aspects of study to reduce the death rates.

In the study for chapter 1, GFP expressing recombinant *Bombyx mori* NPV was constructed and used to visualize the infected organs easily. Larvae were infected with GFP recombinant virus subcutaniously or orally, and the tissues and hemolymph were observed along with time course under fluorescent microscope. The present observation confirmed previous reports that showed the cells of trachea, fat body and hemocytes are heavily infected. I found slightly earlier infection of hemocytes than tissues, and the distinctive difference of infectivity among hemocytes. Although it was confirmed that all kinds of the hemocytes expressed GFP fluorescence, plasmatocytes showed significantly lower expression of GFP compared to others.

In the studies for chapter 2, I observed that the latent period of orally infected larvae was elongated and the rate of survivors increased dramatically (from 0% to 60%) when antiserum against NPV was injected into hemocoel. The level of IgG in hemolymph was checked by Western blotting and Dot Immuno-Binding Assay (DIBA). Although the level of IgG in hemolymph declined gradually, it remained at the detectable level for the whole experimental period. Observation of hemocytes infected with recombinant virus revealed a distinctive difference when anti-NPV serum was applied. The hemocytes of larvae treated with no or unrelated IgG started to express GFP on 2 days post infection. On the contrary, larvae treated with anti-NPV serum suppress the GFP expression. These results suggest that anti-NPV IgG neutralized the virions invaded into hemocoel, and the infection proceeded in only tracheal lymph. In this experiment, I succeeded to block the hemolymph infection conduit, and demonstrated the viral transmission through tracheal lymph system, which has been thought to be the main infection conduit recently. Tracheal system, however, is not enough to attack the host effectively and viral transmission through hemocytes and hemolymph was important in BmNPV infection.

General Introduction:

Sericulture is one of the important industries in rural area of developing countries. Family-level silk production to is recommended to raise the regional family income in India. However, the infection of silkworm, *Bombyx.mori*, with baculovirus *B. mori* Nucleopolyhedrovirus (BmNPV) is one of major obstacles for commercial production of silk in these regions. According to a report, farmers in India usually lose about half of their income by NPV infection (http: 27). Reducing the death of silkworms by viral infection, or delaying the viral attack until spinning will help farmers to increase their income. To regulate the attack of BmNPV infection, it is necessary to understand how the systemic infection in hosts proceeds.

Baculovirus belongs to the family baculoviridae, and has double strand, circular DNA genome. Their genome size is about 130 to 150 kbp (135kbp for BmNPV). In the nature, Baculovirus exists in two forms. One of them is called Occlusion Derived Virus (ODV), which is packaged within the viral polyhedral bodies so called polyhedra. This type of virion is responsible for the inter-insect transmission. The other is called Budded Virus (BV), which has single nucleocapsid in a cellular plasma membrane with several viral proteins like gp64 (Granados and Williams 1986). This type of virion is responsible for the intercellular systemic transmission.

Polyhedra ingested by susceptible larval insect hosts soon dissociates by the alkaline gut juice. Soon after releasing of ODVs, they enter the columnar epithelium and regenerate cells of midgut as first infection site by membrane fusion (ODVs have no spikes of glycoprotein) (Keddie *et al.* 1989). Secondary or the systemic transmission starts from these cells to spread the virus whole body. For systemic infection, scientists propose two main infection conduits. Former one utilize hemolymph channel as the primary infection conduit (Granados and Lawlar 1981, and Keddie *et al.* 1989). There, infected hemocytes and budded virions are thought to float in the hemocoel and infect distanced tissues. The latter one utilizes tracheal lymph channel, which exists between the cuticular lining and the basal lamina of trachea (Engelhard *et al.* 1994). This model proposes that virions come from midgut infect tracheal cells, flow through the lymph system, and infect distanced tissues interconnected by trachea.

The aim of this study is 1) to find the difference of infectivity among hemocytes, which possibly play the important role in the post infectious transmission of BmNPV, and 2) to verify the importance of hemolymph channel for systemic infection by blocking the channel.

Chapter 1: Distinctive difference of hemocytes infection

1. Introduction:

Hemocytes of open circulatory systems are functionally similar to leukocytes of vertebrate rather than erythrocytes. Insects transport oxygen directly from the external environment to tissues through tracheal system, and no oxygen delivering cells exist (Mori *et al.* 1970). Hemocytes play important roles in the insect immune system. They fight against pathogens and parasites by phagocytosis, encapusulation (melanization) and nodule formation etc. (Lackie 1988; Wago 1991; Hoffmann *et al.* 1996; Carton and Nappi 1997, 2001; Lavine and Strand 2002) Roles of hemocytes in immune system have been studied intensively, but the virus infectivity of each hemocyte has not been studied yet. Here, we present that there are marked differences among hemocytes in infectivity against BmNPV infection.

2. Material and Methods:

Experimental animals and cell line:

Larvae of commercial silkworm, *Bombyx mori*, were reared on an artificial diet

(NOSAN) and kept at 25 degree Celsius under a 16-h and 8-h dark photocycle.

The cultured cells of *B.mori*, BmN cell line, was maintained in TC-100 containing 10% of fetal bovine serum as described previously (Katsuma *et al.*, 2004)

Construction of Recombinant virus:

Transfer vector, which containing polyhedrin (polh) gene and egfp gene with hsp promoter was constructed as following method (Fig. 1-1). Gene of *hsp* promoter region was amplified from the plasmid containing *Drosophila. melanogaster* hsp70 promoter with primer PF and PB each containing Nhe-1 or Nco-1 digestion site. Each end of the amplified gene was digested by Nhe-1 and Nco-1.

PF: AAGCTAGCTAGAATCCCAAAACAAACTGG

PB: AACCATGGCAGAGTTCTCTTCTTGTATTC

The fragment of hsp promoter was inserted into the Nhe-1 and Nco-1 region of BmhEPS-1, the transfer vector containing *polh* and *egfp* driven by *immediate early* promotor of BmNPV. BmhEPS-1 was provided from Dr. Katsuma.

The transfer vector, and linear BmNPV DNA, which was linearized at polh

locus (Zhou *et al.*, 1998) were transfected to BmN cells using Cellfectin (invitrogen) to generate the recombinant virus.

The recombinant virus was selected based on GFP expression and polyhedra formation, and purified by plaque isolation three times. The presence of *gfp* at the proper locus was confirmed by PCR amplification of the genomic DNA isolated from the recombinant virus.

The titer of recombinant BV suspension was measured by plaque assay, and 5 x 10^5 PFU / larva of budded virus suspension was used as standard virus solution.

For vBmhspGFP polyhedra production, 10ul of BV suspension was injected into the hemocoel at the third abdominal spiracles of *B.mori* larvae, in the first day of fifth instar. Polyhedra were collected from hemolymph by puncturing the proleg. The polyhedra were pelleted by centrifugation at 3,000 rpm/min, washed 3 times with distilled water, 2 times with 1% SDS, several times with distilled water until no bubbles derived from SDS were observed. Sucrose was added to the final polyhedra solution to promote oral administration. The concentration of polyhedra in the solution was counted on the hemocytometer, and 1 ul of 1 x 10^7 polyhedra / ul solution was used as standard solution to infect single larva.

Infection:

For subcutaneous infection, the BV suspension was injected into the hemocoel at the third abdominal spiracles of *B. mori* larvae. For oral infection, polyhedra of BmNPV were administered with a micro-pipette. Silkworm was held until the droplet of polyhedra suspension was completely ingested. Newly ecdysed fifth instar were used for this experiment.

Observation of GFP expression in hemocytes by fluorescence microscopy:

To avoid the melanization of hemolymph, 1-phenol-2-thiourea-phosphate buffered saline (PBS) solution was prepared (Saturate amount of phenolthiourea was dissolved into PBS). To observe hemocytes, 30-40 ul of larval hemolymph was collected by cutting the projection of tail. Ten ul of phenolthiourea solution was mixed with the hemolymph thoroughly on Parafilm. Then, 10 ul of hemolymph was loaded onto a disposable hemocytometer, C-chip (iNCYTO), and the fluorescence of hemocytes was observed immediately under fluorescence microscope, XB-51 (Olympus). Each type of hemocyte was classified based on the following standard criteria (Mori *et al.*, 1970)

Classification of hemocytes:

Hemocytes were classified by photo-microscopy in the absence of staining. The following criteria, which were modified based on the past reports (Nittono 1960; Mori *et al.* 1970, Akai and Sato 1973), were used for classification.

Prohemocyte relatively large nucleus to its cell size (6-12um in diameter). Classified under fluorescent microscope only after nuclear staining with formalin and propidium iodide

Granulocyte the most abundant hemocyte in hemolymph, which has sphere shape and contains tiny granules in cytoplasm. Sometimes, granulocytes and prohemocytes are difficult to distinguish. Only hemocytes containing distinctive granules are counted as granulocytes, and the indistinguishable hemocytes were counted as unknown.

Plasmatocyte irregular spike-like projection on the surface of cell, forms spindle outlooking and sticking nature toward the wall of

hemocytometer.

Spherulocyte contains small spheres in its cytoplasm, and this makes the wave-like outlines of cells

Oenocytoid easy to classify for its relatively large in size (12-25 um in diameter), fragile nature and opaque appearance compared to other hemocytes

Observation of tissue samples under fluorescence microscope:

Biopsy of infected larvae was made through the dorsal cuticle along the length of the body. Tissues (midgut, fat body, trachea, gonads, silk glands and neurons) were exercised, and washed in cold PBS. The samples were immediately observed under fluorescence microscope.

Results:

For tissue observation, the present observation confirmed previous reports (Rahman and Gopinathan. 2003, and Barret *et al.* 1997) that showed the peritracheal cells attached to each tissue, trachea, fat bodies and hemocytes expressed high GFP expression (Fig. 1-2). GFP expression of hemocytes was observed a little earlier than that of other tissues (Data not shown). In this observation, the difference of infectivity of hemocytes was observed. GFP fluorescence observation of hemocytes clarified that all kinds of hemocytes had been infected (Fig. 1-3). However, it was very rare to see plasmatocytes emitting fluorescence, especially in the early phase of infection.

When larvae were infected orally, GFP expression started to be seen between 36 to 48 hour post-infection (hpi) for granulocytes and oenocytoids (Fig. 1-4). But the expression of plasmatocyte was observed only after 60 hpi. Both granulocytes and oenocytoid get over 50% of their GFP expression after twelve hours since GFP expression was observed, and the ratios of hemocytes expressing GFP were reached more than 90 % finally. On the contrary, the ratio of GFP expression of plasmatocyte was relatively low in comparison with than the other cell types, and showed little growth between 60 and 84 hours. Only less than 20% of plasmatocytes expressed GFP by 84 hpi. But the percentage of plasmatocytes expressing GFP suddenly increased

between 84 and 96 hpi. About 60% of plasmatocytes expressed GFP finally. The last observation of hemocytes for orally infected larvae was performed at 96 hpi due to the emerging of massive amount of polyhedra in hemolymph after 108 hpi.

When larvae were infected subcutaneously, 1.4% of granulocytes started to express GFP at 12 hpi (Fig. 1-5). Then, GFP expression of spherulocytes and oenocytoids arose at 24 hpi. The ratios of GFP expression for granulocytes, spherulocytes and oenocytoids kept rising for whole experimental period. On the other hand, GFP expression of plasmatocytes was observed only after 36 hpi, and the ratio did never go over 10% for whole experimental period. The observation of hemocytes for subcutaneous infection was stopped at 60 hpi due to the emerging of polyhedra in hemolymph after 72 hpi.

Discussion:

Granulocytes, the most abundant circulating hemocytes, tend to be in cluster and infected earlier in both oral and subcutaneous infection. Granulocytes have high phagocytosis activities. So, it is possible that granulocytes ingested virions by phagocytosis, and the phagocytosed virions might escape from the decomposing process inside granulocytes.

Plasmatocytes have similar function to platelet cells in vertebrates. In both oral and subcutaneous infection, plasmatocytes showed very low BmNPV infectivity. Plasmatocytes might have different composition of membrane proteins, which show relatively low affinity to viral protein like gp64 compared to other hemocytes. The difference of phagocytosis or pinocytosis ability among hemocytes might influence the infectivity of themselves toward virus like BmNPV.

Ling *et al.* (2005) recently reported that hematopoietic organ of *B. mori* contains only proleukocytes and oenocytoids based on their observation with acridine orange and propidium iodide (PI) staining. They classified proleukocyte as unstained cells with PI but stained with acridine orange, and oenocytoid as stained cells in opposite manner. Because of the fragility of cell membrane, oenocytoids are stained with PI in a living form. In their research, the sensitivity of undifferentiated oenocytoids

toward acridine orange was not concerned, and the researchers might count undifferentiated oenocytoids as proleukocytes. So it is possible that the two types of cells they observed in their study are proleukocytes include undifferentiated oenocytoids, and "differentiated" oenocytoids. According to the old report of hematopoietic organs, only proleukocytes and plasmatocytes are observed under photomicroscope (Mori *et al.* 1970). If their observation is correct, it is reasonable to think that the type of blast cells for plasmatocyte and the rest of fours are totally different, and this affects the difference of hemocyte infectivity towards BmNPV.

Our further attempts to verify the factors involved in the mechanisms of plasmatocyte infectivity were not successful because of technical difficulties. To analyze hemocytes molecularly, it is inevitable to separate hemocytes into each type. But contemporary, there was no way to separate cells in a high accuracy. Even some methods, by which we can separate hemocytes in low accuracy requires the step of cell staining, which disturb the GFP fluorescence of infection.

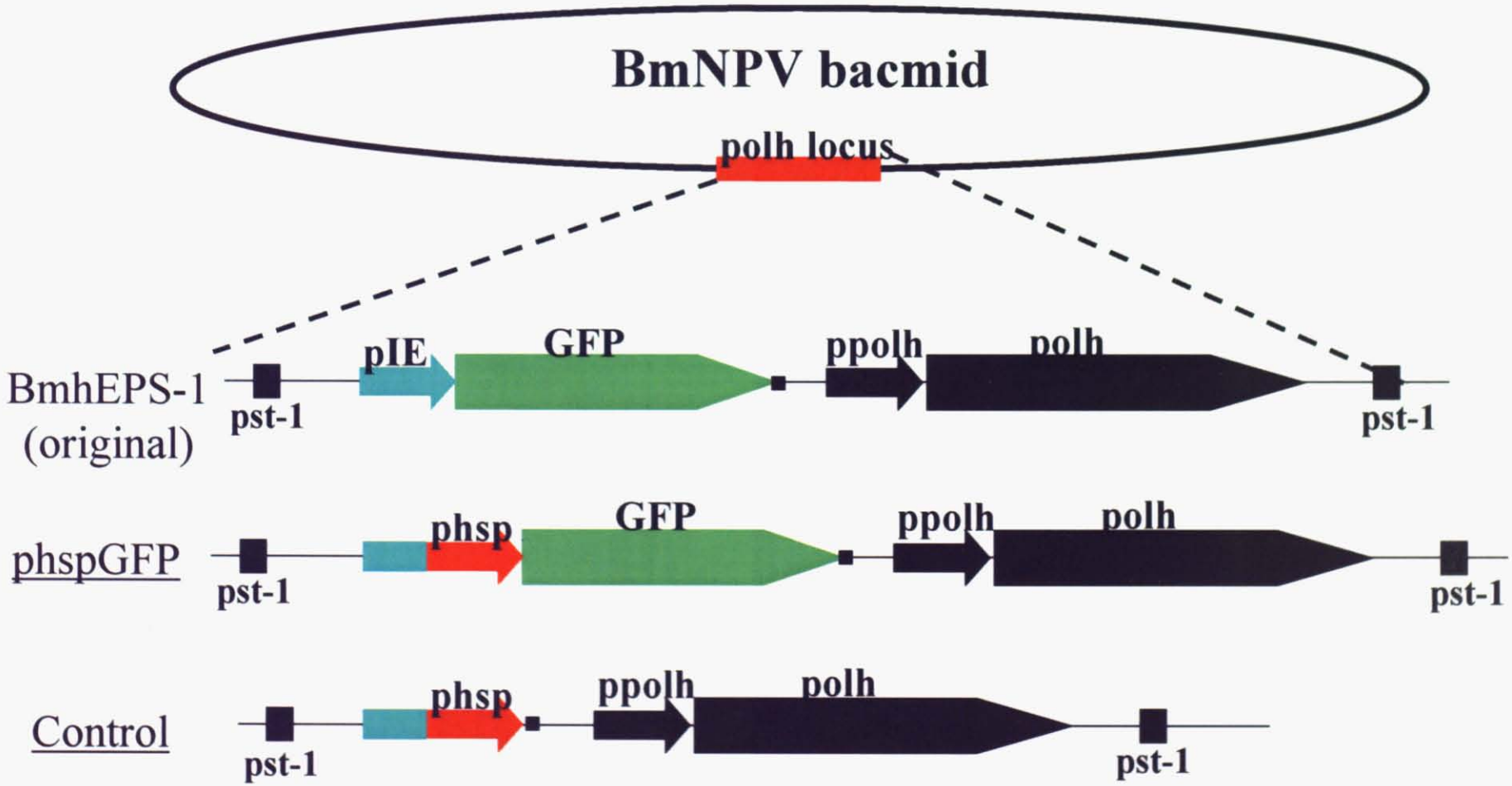

Fig. 1-1 Construction of recombinant virus

Transfer vecter phspGFP and its GFP negative control was constructed and co-transfected with linear BmNPV bacmid to get the recombinant virus

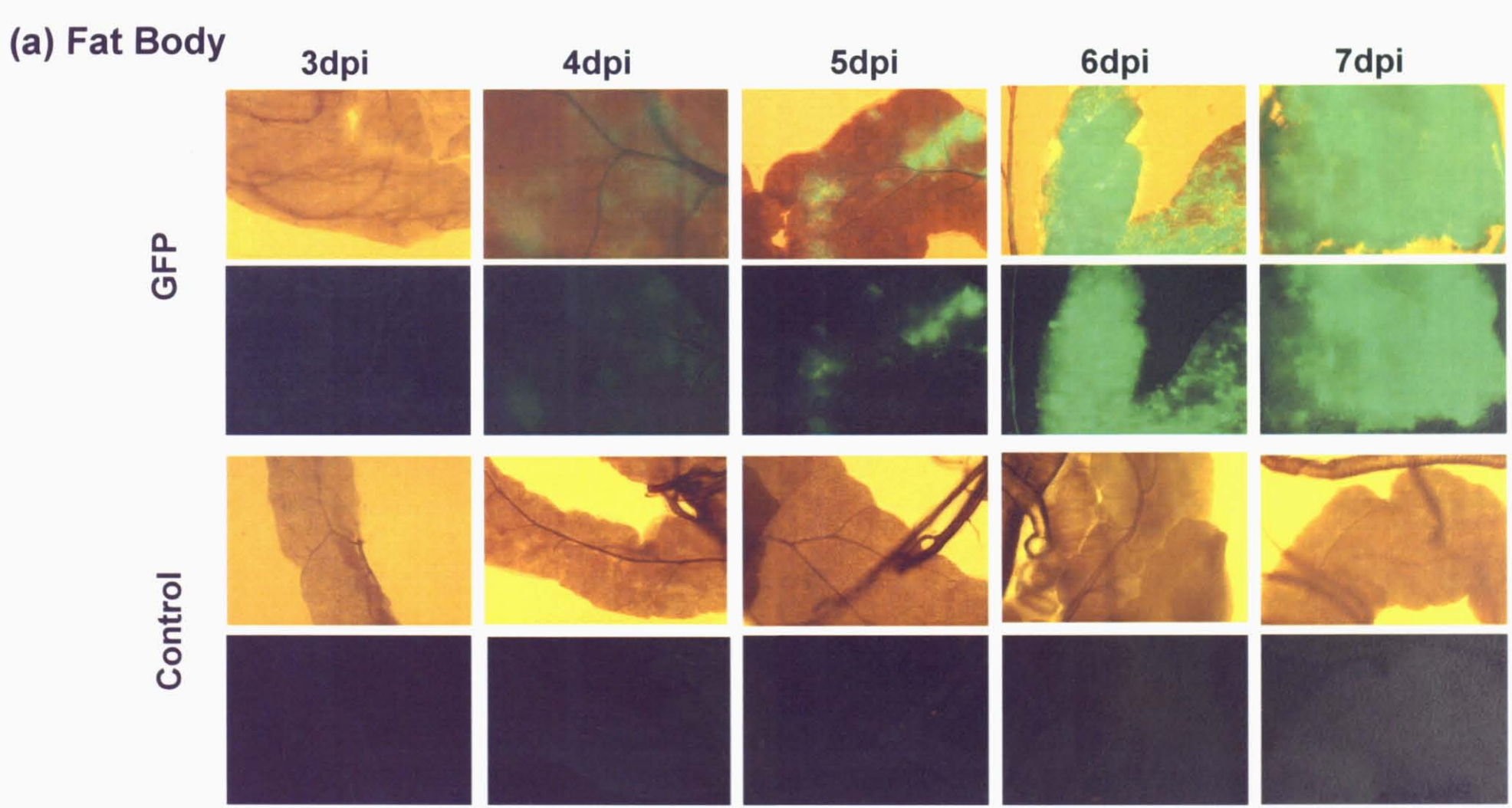

Fig. 1-2. Observation of GFP fluorescence in selected tissues of *B. mori* infected with *Bm*NPV – hspGFP at several time points. Larvae (fifth instar, day 1) were infected orally with the viral polyhedral bodies and were reared on artificial diet at 25 °C. Tissues were dissected out of 5 larvae each day, and photographed with an Olympus BX-51 fluorescence microscope. (a) Fat body, top panels of GFP: marge images of bright field and GFP fluorescence between 3 and 7 dpi. Bottom panels of GFP: GFP fluorescence of the same image

(b) Trachea

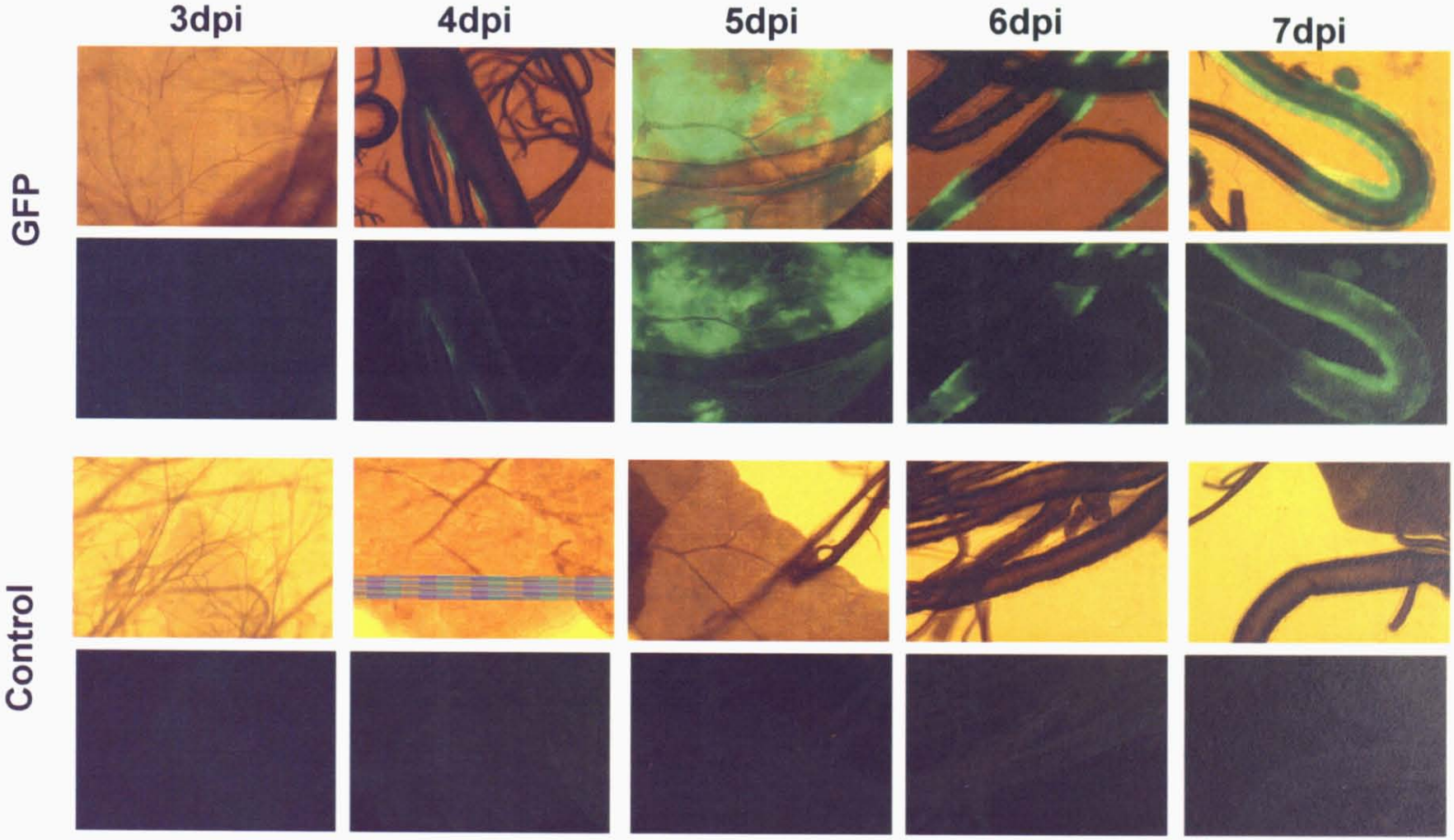

Fig. 1-2. (*Continued*). (b) Trachea, top panels of GFP: marge images of bright field and GFP fluorescence between 3 and 7 dpi. Bottom panels of GFP: GFP fluorescence of the same image. Note the discontinuity of fluorescence in trachea on 6 and 7 dpi

(c) Neuron

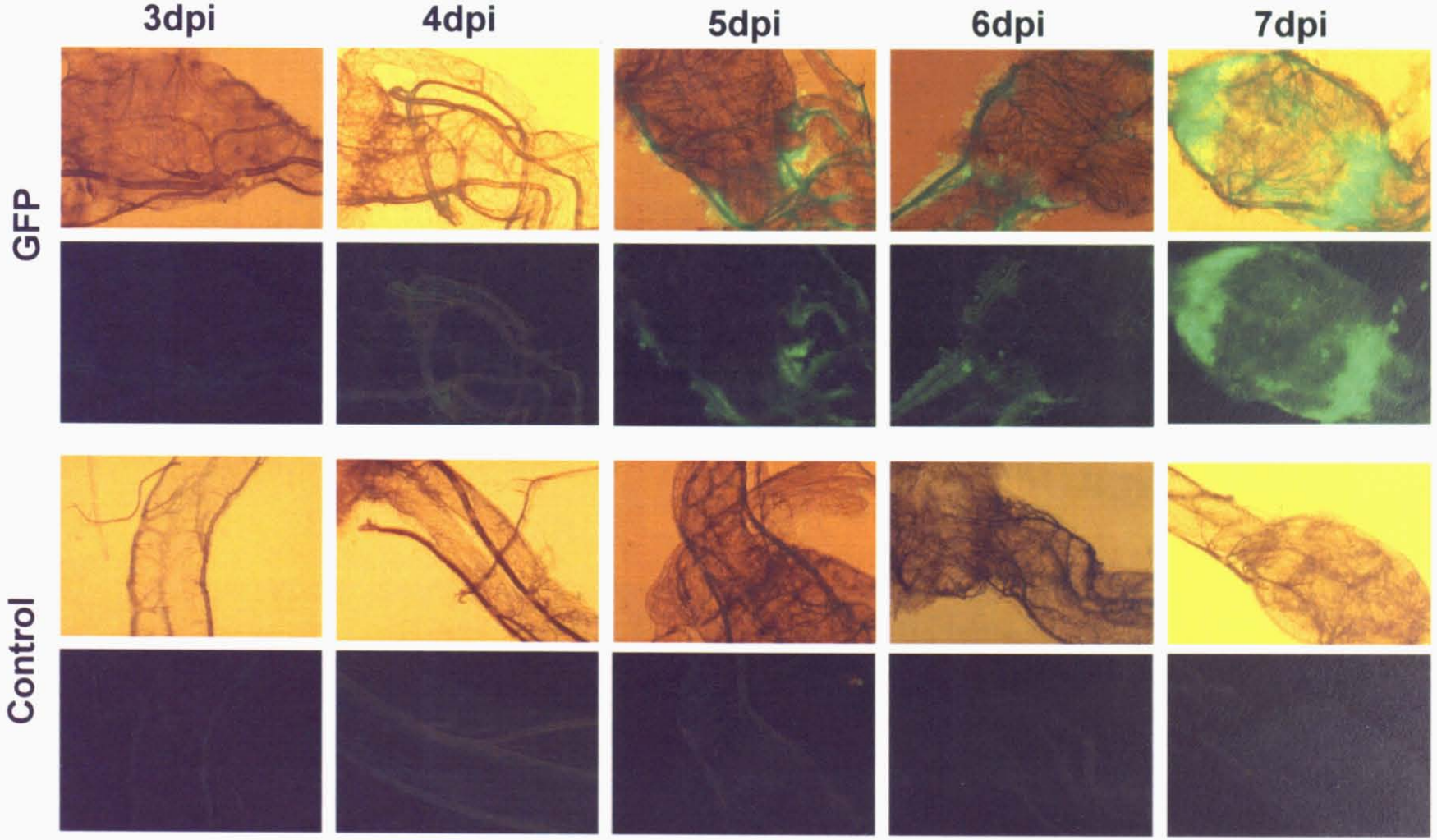

Fig. 1-2. (*Continued*). (c) Neuron, top panels of GFP: marge images of bright field and GFP fluorescence between 3 and 7 dpi. Bottom panels of GFP: GFP fluorescence of the same image.

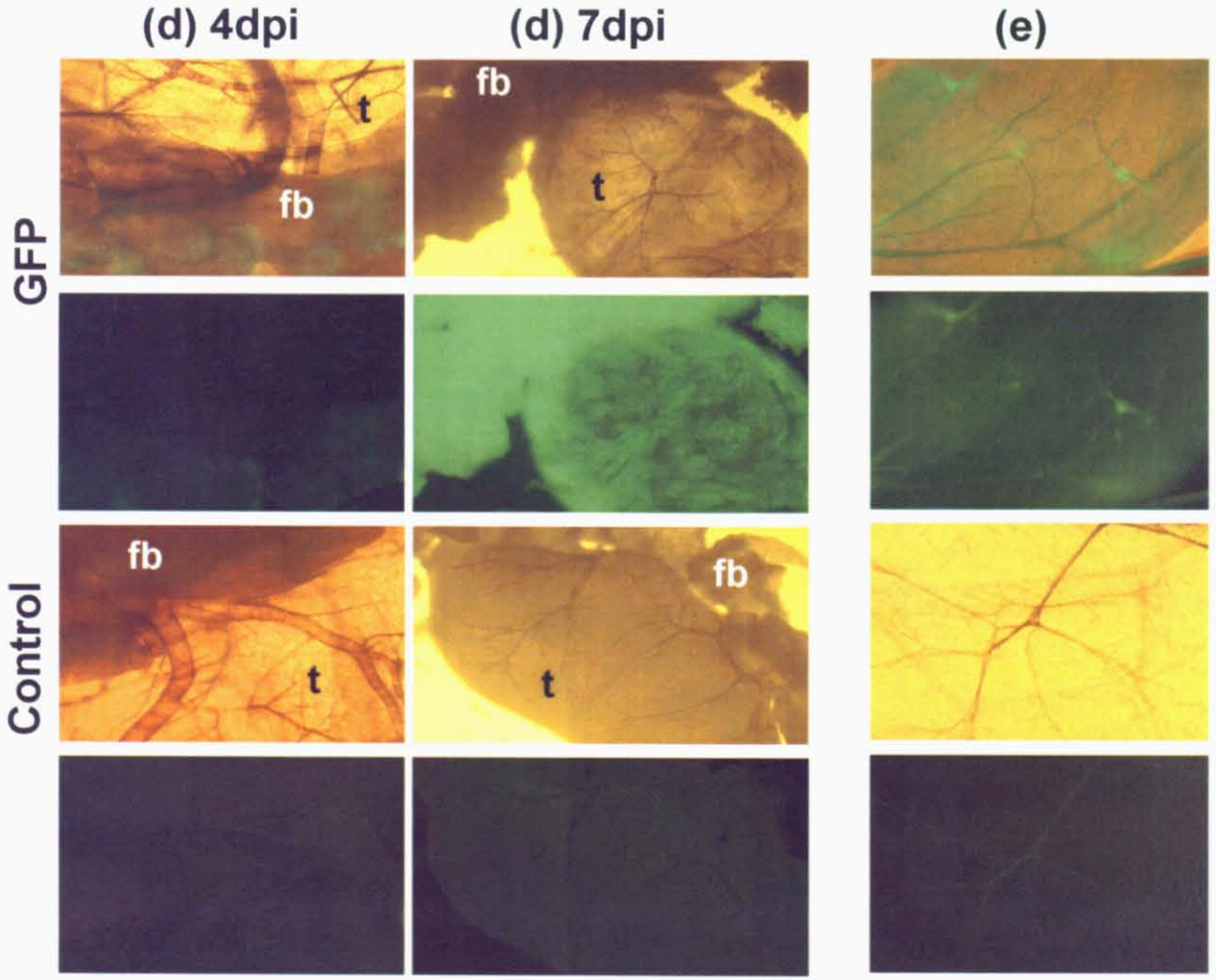

Fig. 1-2. (*Continued*). (d) Trachea on 4dpi and 7dpi top panels of GFP: marge images of bright field and GFP fluorescence on 4 and 7dpi. Bottom panels of GFP: GFP fluorescence of the same image. (e) posterior silk gland on 7 dpi, top panels of GFP: marge images of bright field and GFP fluorescence on 7dpi. Bottom panels of GFP: GFP fluorescence of the same image. t: testis, fb: fat body

(f) Malpighian tubule (7dpi)

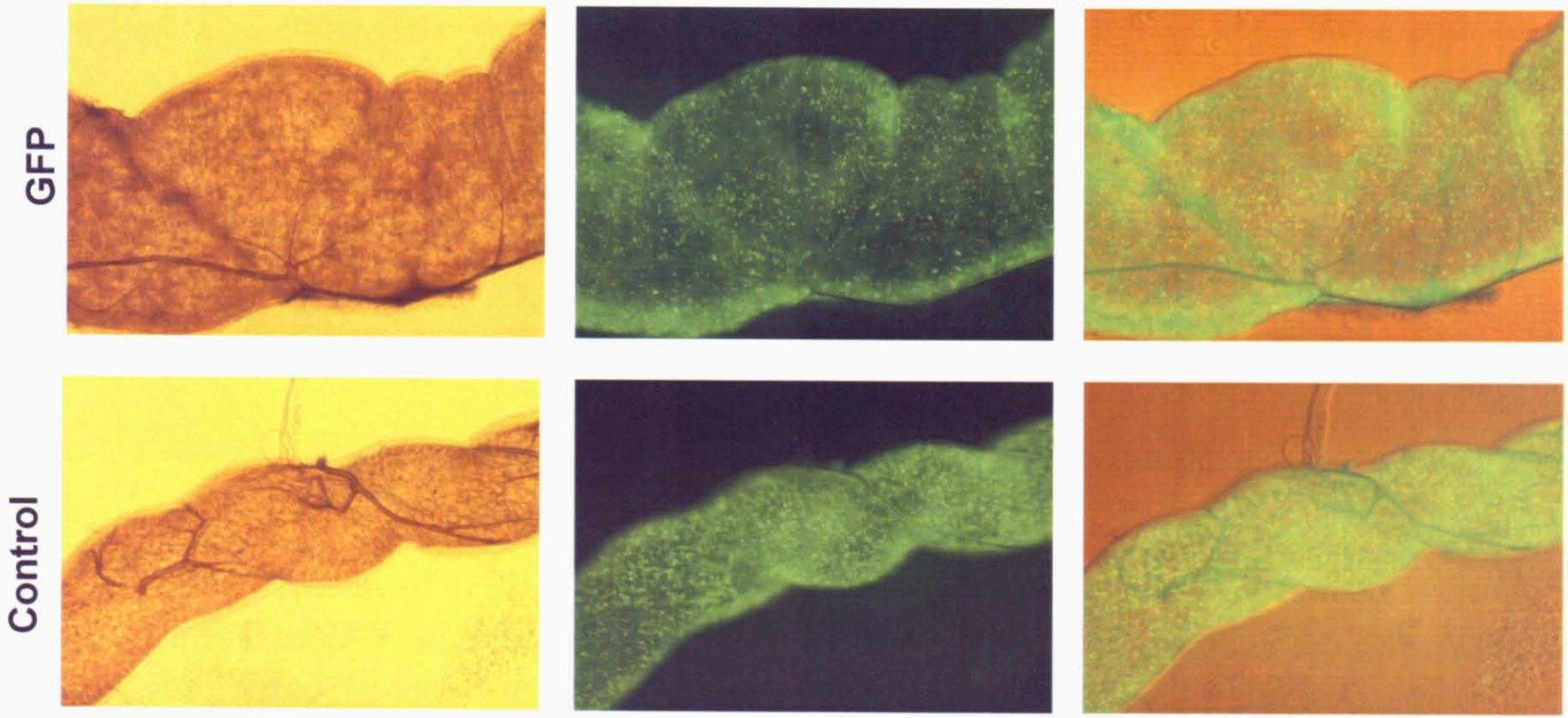

Fig. 1-2. (*Continued*). (f) Malpighian tubule, left panels: bright field on 7 dpi. Middle panels: GFP fluorescence of the same image. Left panels: merge images of bright field and GFP fluorescence of the same image.

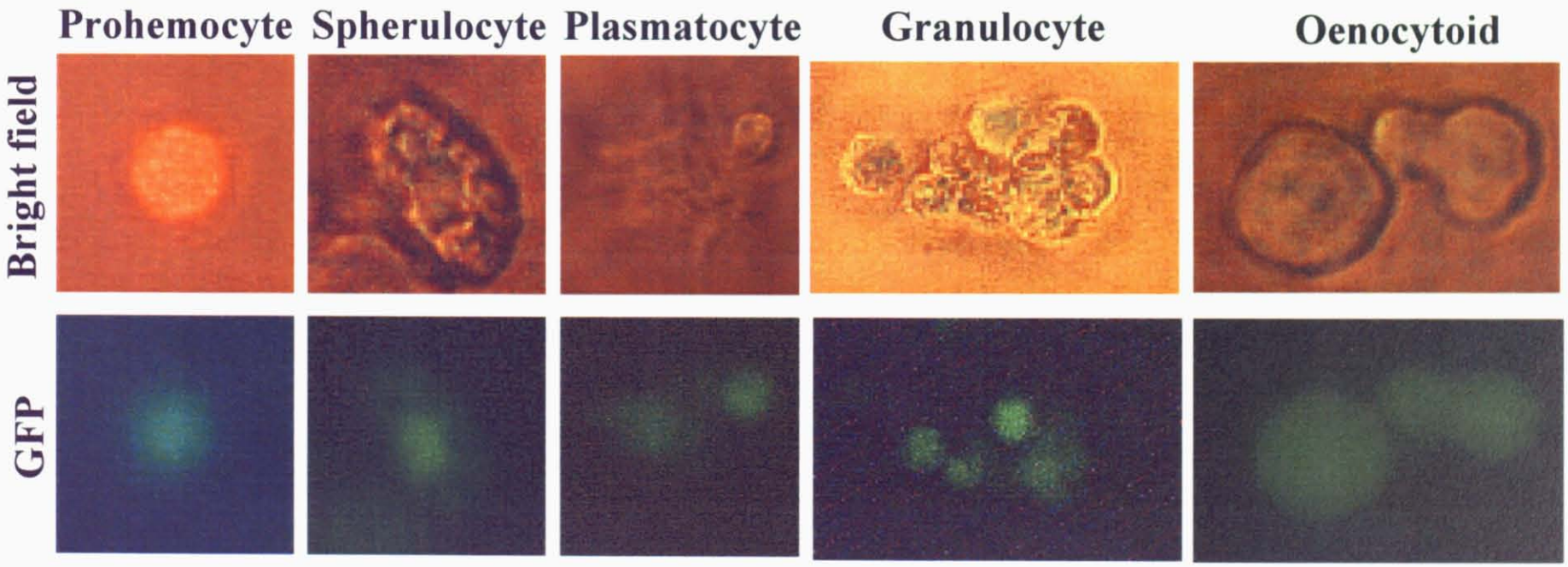

Fig. 1-3 Observation of GFP fluorescence in hemocytes of B. mori infected with GFP recombinant BmNPV. Hemolymph was collected at 60 dpi from subcutaneously infected larvae, and photographed with BX-51 fluorescence microscope (Olympus). The photograph of prohemocytes was taken after formaldehyde processing followed by propidium iodide staining.

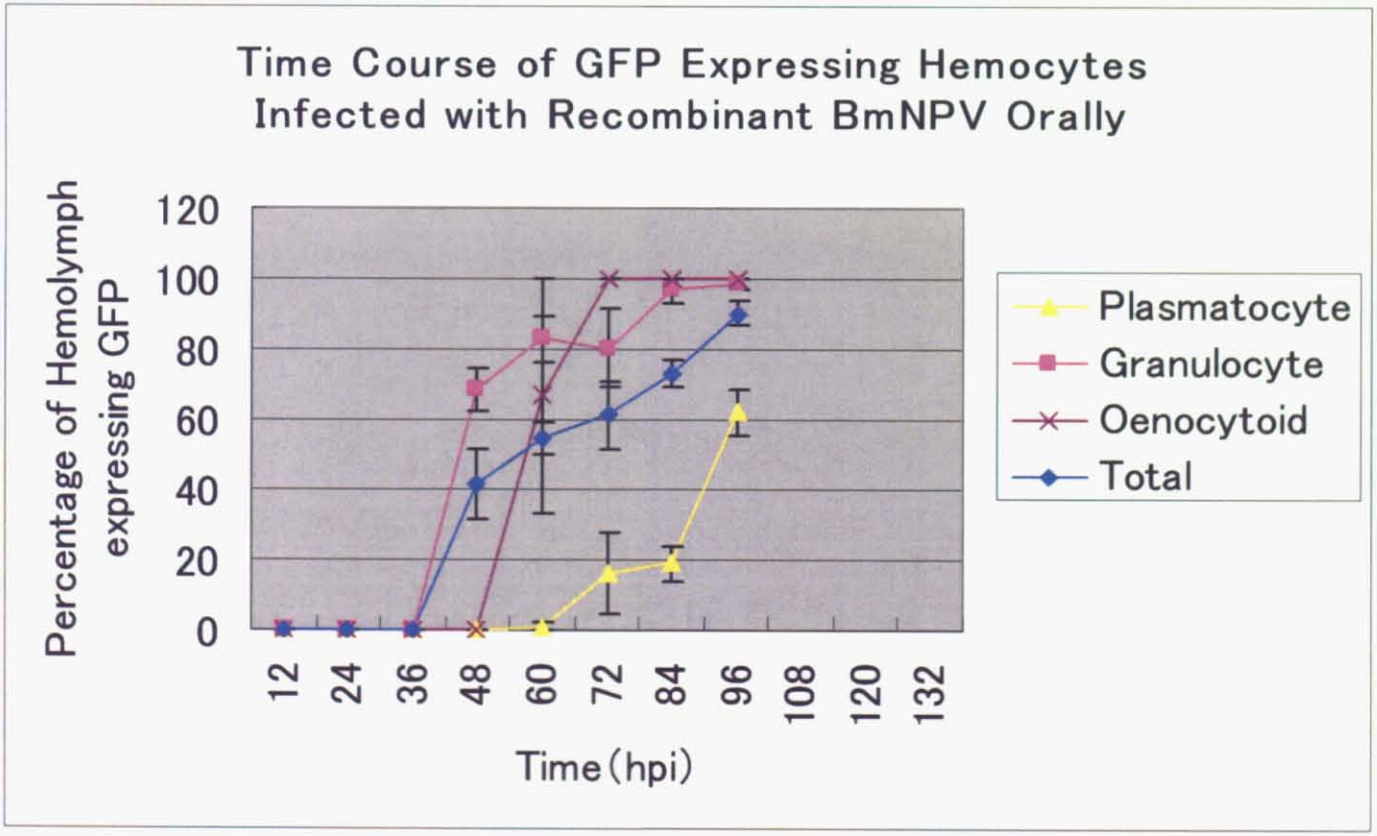

Fig. 1-4 Time course of GFP expression in hemocytes collected from orally infected larvae. Hemolymph was collected every 12 hours since the virus are administered orally till the emerging of polyhedra in hemolymph (108 dpi).Indistinguishable hemocytes, which are mainly consisted by granulocytes and pro-hemocytes were omitted. Spherulocytes were not counted this time. Hemolymph was collected from five larvae at each time point, and the fluorescence was observed separately.

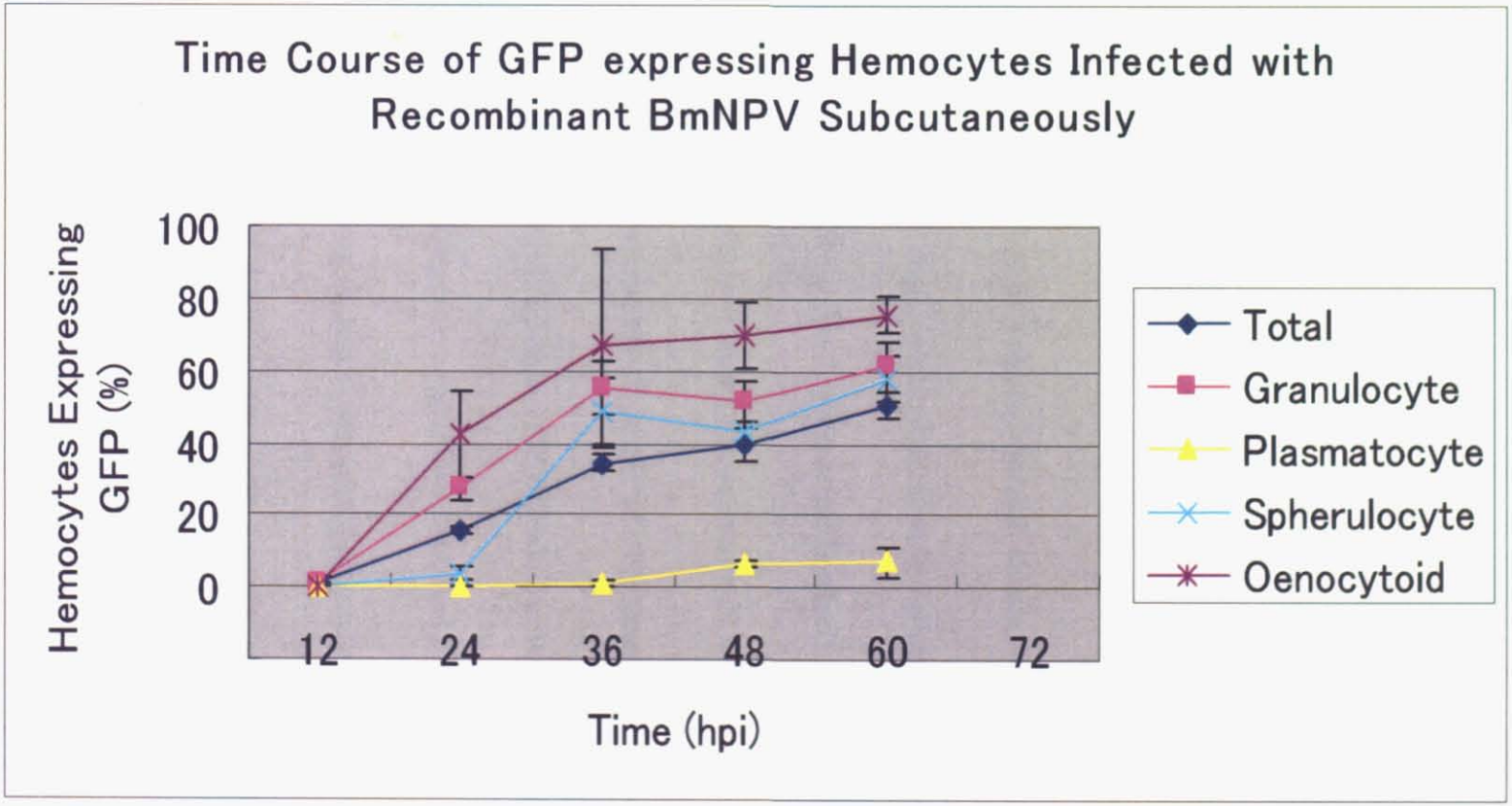

Fig. 1-5 Time course of GFP expression in hemocytes collected from subcutaneously infected larvae. Hemolymph was collected for every 12 hours since the virus solution were injected into hemocoel till the emerging of polyhedra in hemolymph (72 dpi). Indistinguishable granulocytes and pro-hemocytes were counted as unknown hemocytes and omitted. Hemolymph was collected from five larvae at each time point, and the fluorescence was observed separately.

Chapter 2: Shutting Down the Hemocoel Infection Conduit Temporally with Neutralizing Antibody and Proving the Importance of Open Circulatory Systems for Systemic Viral Infection

1. Introduction:

Understanding the systemic mechanism of how virions spread around the whole body and how it triggers high and sudden host's mortality is one of the important aspects to study. Once, it is thought that hemocytes were the principal factors to spread the infection whole body (Granados and Lawler 1981, and Keddie *et al.* 1989). But some contradictions occurred about the existence of basal lamina. Basal lamina is secreted layer virtually surround all kinds of the tissues in insects (the only exception is hemocytes)(Reddy and Locke 1990). Basal lamina has selective permeability and is a barrier from baculovirus entry (Hess 1987, and Tanada and Hess 1991). Because of the existence of this barrier, it is thought that neither can BV infect other tissues nor can get out of the first infection site, midgut, to the hemocoel of the host.

To resolve this enigma, one systemic infection model utilizing tracheal system was proposed by Engelhard *et al* (1994). They suggested that virions are transmitted across the basal lamina through the infected tracheoblast, which can successfully

invade and penetrate the barrier. Theoretically the idea of Engelhard, which is based on the observation of tracheoblast infection made it possible to explain how hemocytes infected. They suggested that tracheoblasts play a role of intermediate factor of midgut-hemocytes infection. Moreover, based on the study of tracheal structure by Reddy and Locke (1990), they suggested that virions entered the tracheal lymph channel utilize the tracheal lymph channel for rapid spread of virus infection throughout the host, but not hemocoel.

Still it is not clear which is more important for the systemic infection, hemolymph or tracheal lymph. And the degree of the importance of these factors varies depend on the species of both host and virus (Trudeau *et al.* 2000).

In this study, we found that the latent period of BmNPV was elongated and the survival rate increased dramatically when rabbit-derived anti-NPV antiserum was applied into hemocoel of silkworm larvae. This suggests that the antibody worked as neutralizing agent against virions invaded into hemocoel. We propose that tracheal lymph conduit is not enough for systemic infection by blocking the invasion of virions into hemocoel with neutralizing antibody.

2. Material and Methods:

Experimental animals and cell line:

Larvae of commercial silkworm, *Bombyx mori,* were reared on an artificial diet and kept at 25 degree Celsius under a 16-h and 8-h dark photocycle.

The cultured cells of *B.mori*, BmN cell line, was maintained in TC-100 containing 10% of fetal bovine serum as described previously (Katsuma *et al.*, 2004)

Recombinant virus:

The same strain of virus used in chapter 1 was used for this experiment.

Infection:

For subcutaneous infection, the BV suspension was injected into the hemocoel at the third abdominal spiracles of *B. mori* larvae.

For oral infection, polyhedra of BmNPV were administered with a micro-pipette by putting a droplet of polyhedra solution on the mouth of silkworm. Newly moulted fifth instar larvae were used for this experiment.

Antiserum:

Anti-sera used for this experiment were prepared by injecting *B. mori* cypovirus (BmCPV), or non-occluded virions of BmNPV into rabbits (Nagata *et al.*, 1997). Fifty ul of serum was injected to each larva at 6-hour-post-infection (hpi).

Survivor's rate:

To measure the transition of survivor's rates, 1 x 10^7 of polyhedra per larva of the recombinant virus were administered orally. At six hour after the oral administration, 50 ul of the serum containing anti-CPV or anti-NPV antibody was injected into hemocoel (15 larvae per each cohort). To determine whether the serum injection itself affects the survivor's rates, serums of anti-CPV or anti-NPV were injected to uninfected larvae (7 larvae per each cohort). The numbers of survivors were checked and recorded daily.

Hemolymph collection:

Hemolymph samples were collected at following time course (anti-CPV: 2, 4 and 6 dpi; anti-NPV: 2,4,6,8,10 and 12 days post infection (dpi)) by cutting the projection of tail. As control, hemolymph of uninfected and antiserum-free larva is

prepared.

Western blotting and Dot Immunobinding Assay (DIBA) to detect IgG in hemolymph:

Western-blotting test and DIBA for hemolymph were performed to check whether the IgG injected into hemocoel were remaining at each time course. For Western blotting, proteins in collected hemolymph were separated on polyacrylamide gel and transferred onto PVDF membranes using semidry electric transfer. DIBA was carried out as described by Hawkes *et al.* (1982) without the step of first antibody conjugation for the purpose of detecting IgGs remaining in the hemolymph. Antibodies in the hemolymph were detected using anti-rabbit IgG conjugated to horseradish peroxidase (BioRad), followed by naphtole-hydroxy peroxide reaction.

Observing the viral invasion into hemocoel:

Invasion of virions into hemocoel was monitored with GFP fluorescence microscopy. Larvae were infected with GFP recombinant virus, and treated with antibody-containing serum in the same manner as above. Hemolymph was collected

from three larvae every 24 hour.

For GFP fluorescence microscopy of hemocytes, 40 ul of larval hemolymph was collected. The collected hemolymph was immediately mixed with 10 ul of phenylthiourea-PBS solution to avoid melanization. Then, 10 ul of hemolymph was loaded onto a disposable hemocytometer, C-chip (iNCYTO), and the percentages of hemocytes expressing GFP against the total number of that in the counter square were counted immediately under fluorescent microscope, XP-51(Olympus).

Observation of tissue samples under fluorescent microscope:

Infected larvae were dissected longitudinally through the dorsal cuticle. Tissues (the midgut, fat body, trachea, gonads, silk glands and neurons) were exercised, and washed in cold PBS. The samples were immediately observed under fluorescence microscope.

3. Results:

Survivor's rate:

Anti-CPV or anti-NPV containing serum (v = 50 ul) was injected to uninfected larvae to check the influence of injecting serum to silkworm larvae (Fig. 2-1). The survivor's rate marked 100%. Although injecting 50 ul of serum looks quite tough for this small host (1g / larva), it was confirmed that injecting this amount of serum itself is not harmful. Applying massive amount of serum, like 100ul and 200ul, induced malfunction of digestive tract and unexpected death (data not shown).

The NPV-infected larvae treated with anti-CPV started to die on 4 dpi (Fig. 2-1), and all larvae in this cohort died by 8 dpi, showing polyhedra in hemolymph. The cuticule of dead larvae, was fragile by the post-mortal viral protease activity.

On the contrary, the infected larvae treated with anti-NPV kept high survival rate (Fig. 2-1). Although some portion of larvae died for NPV infection between 9 and 12 dpi, the survivor's rate at the end of this experiment was about 60 %.

By 12 dpi, the survivors treated with anti-NPV started spinning, and their epidermis turned to be softer and semitransparent for ecdysis. These changes are similar to the diagnosis of late phase of NPV infection. Because it was difficult to diagnose the cause of death after 13dpi, we stopped the observation on 12 dpi.

Western blotting and Dot Immunobinding Assay (DIBA) to detect IgG in hemolymph:

In the western blotting test, the bands of heavy and light chains of IgG injected into hemolymph were detectable by 4 or 6 dpi (Fig. 2-2). But it became difficult to detect the antibody after 8 dpi. Since the IgG was denatured in the step of SDS and mercaptoethanol, detection efficiency may be low to detect. To see the changes of IgG level in hemolymph, DIBA was tested (Fig. 2-2). Although the amount of antibody declined as days proceed, both anti-CPV and anti-NPV IgG was detectable during the whole period of this test (12dpi).

Observation of the viral invasion into hemocoel:

Both of the larvae treated with no or unrelated antiserum showed similar pattern of GFP expression in hemolymph, expressing the GFP in hemocytes started to be observed from 2 dpi, and all larvae showed GFP expression by 3 dpi (Fig. 2-4). After 3 dpi, the GFP expression in hemocyte was observed in every larva. For the infected larvae treated with unrelated IgG (anti-CPV), the percentages of hemocytes expressing GFP gradually increase by 5 dpi in the similar manner to the

untreated larvae (Fig. 2-5).

On the other hand, GFP fluorescence was not observed in the hemocytes of the infected larvae when they are treated with anti-NPV (GFP fluorescence was observed only on 6 days) (Fig. 2-4 and 2-5).

Observation of tissue samples under fluorescent microscope:

The expression of GFP in tissues started to be observed around 60-72 hpi if the infected larvae were not treated with antibody (Fig. 2-6 e and f, or Fig 1-2) or treated with unrelated antibody. When the infected larvae were treated with anti-NPV antiserum, expression of GFP in tissues was delayed (Fig. 2-6 b and d). GFP fluorescence was observed on 9 dpi, but it was quite weak (Fig. 2-6 b). For the anti-NPV treated larvae, the fluorescence of tissues increased from 9 dpi to 12 dpi (Fig. 2-6 b and d), even though the fluorescence of hemocytes was not observed (Fig. 2-4 and 2-5). No tracheoblast infection was observed (Fig. 2-6 b and d). Normally, the cells around tracheae or branching point of that tend to get infection at first, and it seems like infection spreads from these specific cells (Fig. 2-6 e and f). But I could not find any fluorescence at all around these points for anti-NPV treated tissues, and it seemed like the whole cells in the same tissue increased the

fluorescence without inclination (Fig. 2-6 b and d).

The BmNPV infection to tissues was comfirmed not only by GFP fluorescence, but also by the observation of polyhedra in tissue cells and the swelling of the epitherium cells surrounding cuticular of trachea by photo-microscopy.

4. Discussion:

When the tissue samples and hemocytes collected from the same larvae was observed, GFP expression of anti-NPV treated larvae was observed only in tissue samples, but not in hemocytes (Fig. 2-4, 2-5 and a-d of 2-6). The intensity of GFP in tissue samples was increased between 9 and 12 dpi. This suggests that the viral infection was progressed in the tissues. Although the expression of GFP in the hemocytes of anti-NPV treated larvae was observed on 6 dpi, the fluorescence of hemocytes is likely to be suppressed for whole experimental period. This strongly suggests that the hemocoel infection conduit was blocked by anti-NPV antiserum, which neutralized the virions emerged into hemolymph system, and allowed only in tracheal lymph system to proceed the infection. In the absence of virions in hemolymph, virions infected the tissues were possibly transported through the tracheal lymph systems, which is attached to and connecting all tissues.

In the observation of survivor's rate, the latent period of orally infected larvae was elongated from 2 day to 8day, and the rate of survivors increased dramatically from 0% to 60% when anti-NPV antiserum was injected into hemocoel. This result suggests that tracheal lymph system only was not enough to kill larvae effectively, and hemolymph system is necessary for systemic infection. Moreover, the infected larvae

treated with anti-NPV started to spin to prepare for pupation on 9dpi. BmNPV has a gene of Ecdysteroid UDP-glucosyl transferase (*egt*)(O'Reilly *et al.* 1989). The translated product of *egt* modifies the host hormone ecdysone to render it inactive. Therefore, inhibition of spinning and putation is disturbed in larvae infected with BmNPV. But the infected larvae treated with anti-NPV started spinning. This means that the EGT produced by infected cells was not enough to inhibit the host ecdysone activity at the specific time.

From these phenomena, it can be conjectured that virions cannot reach enough or threshold level to carry out its systemic infection when they are replicated only in tracheal lymph system, which means hemolymph system is necessary for systemic infection. Hemocytes in hemolymph might carry virion to various tissues, or might be used to amplify virions.

Before the tracheal infection conduit was proposed, hemolymph system had been thought to be the main factor of systemic infection. But after the tracheal systemic infection model was proposed, scientists tend to follow this idea without any direct demonstration. The present experiments demonstrate that tracheal lymph system, which is thought to be the main infection conduit in recent studies, is not enough to attack the host effectively

When the hemocoel conduit was shut down by anti-NPV IgG, GFP expression of tracheoblast was not found under fluorescence microscope. But the GFP expression of tissues was observed without tracheoblast GFP expression. The absence of tracheoblast GFP expression suggests that the tracheoblasts are infected from hemocytes. In the study for chapter 1., GFP expression of hemocytes was slightly earlier than that of tissues. This observation suggests that tracheoblast was infected from hemocytes or virions floating hemocoel, but not infected through tracheal lymph system. It is possible that infection of hemocytes is necessary for distanced infection within a host. This idea supports the importance of hemolymph system in systemic infection.

The results in the present study suggest that 1). The amount of virions replicated and transported in tracheal lymph system was not enough for systemic infection 2). The hemocyte infection and amplification of virion in the hemocytes are necessary to kill the host properly and 3) Hemocytes work as the principal vehicles for the spread of infection within the host larva. Further study, however, is required to prove the importance of NPV infection through the hemolymph system rather than tracheal system.

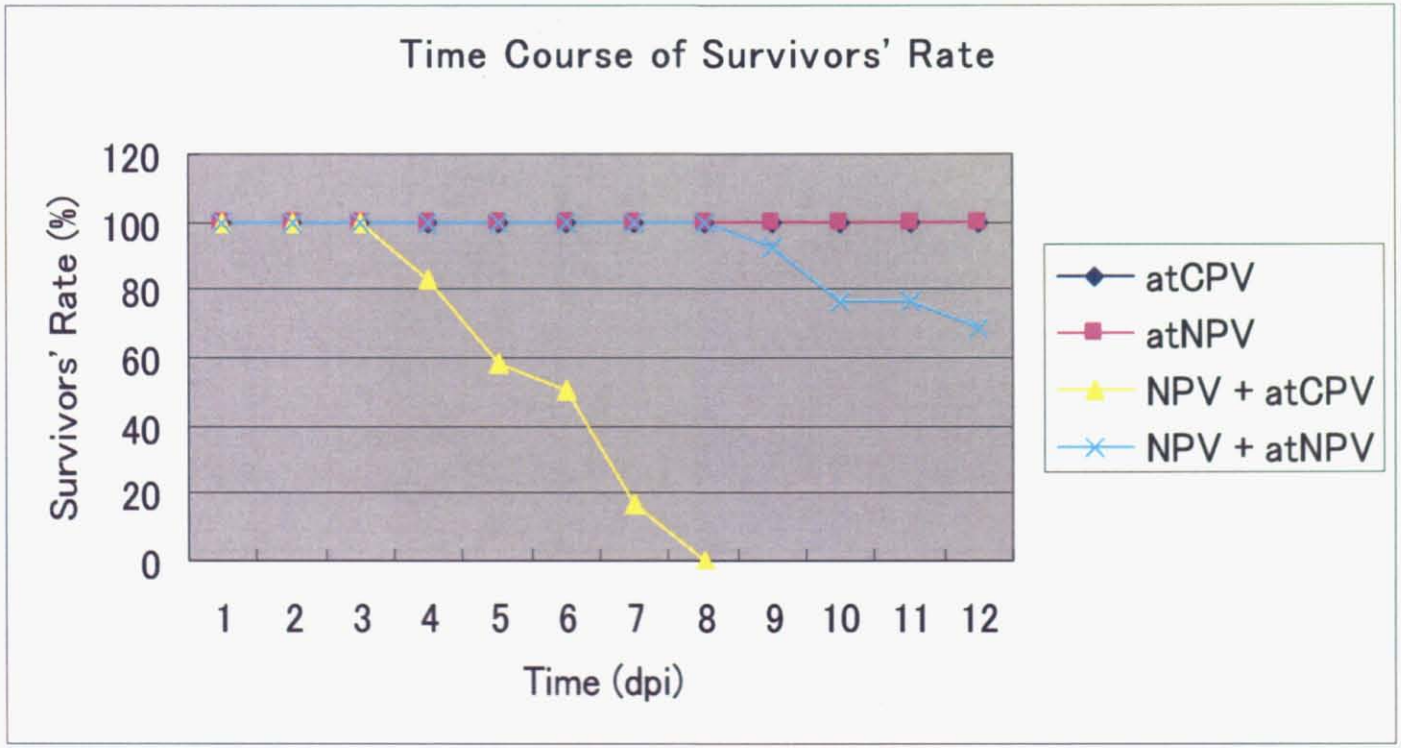

Fig 2-1. Time course of post-infectious surviving rate. atCPV or atNPV represents the larvae orally treated with anti-CPV or anti-NPV anti-serum. NPV + atCPV or atNPV represents the larvae orally infected to BmNPV and treated with anti-CPV or anti-NPV anti-serum at 6 hpi. 15 larvae were prepared for each cofort.

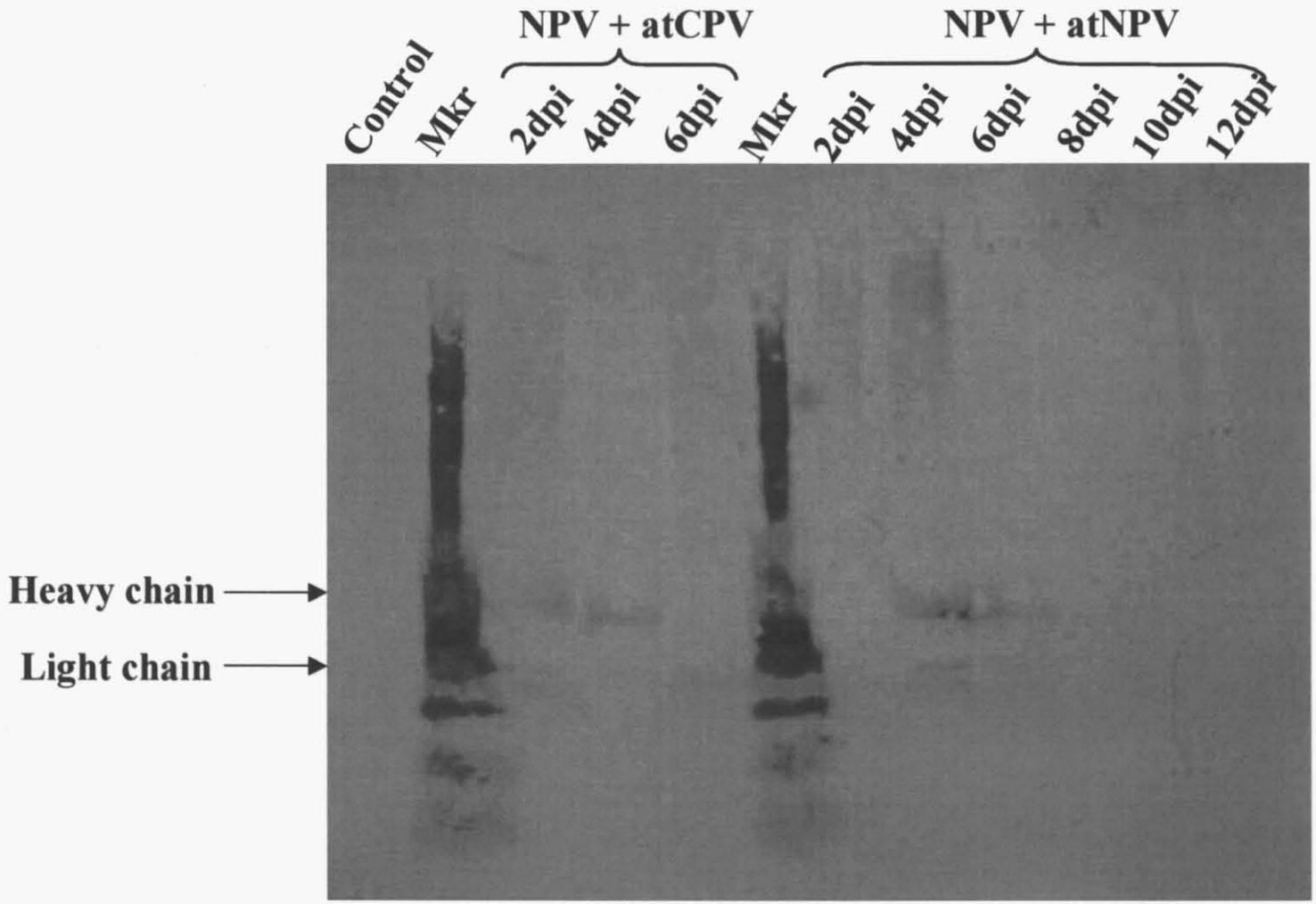

Fig. 2-2 Western blotting test to check the level of IgG left in the hemocoel.
Secondary antibody was treated to the membrane directly, and detected with naphtol and H_2O_2

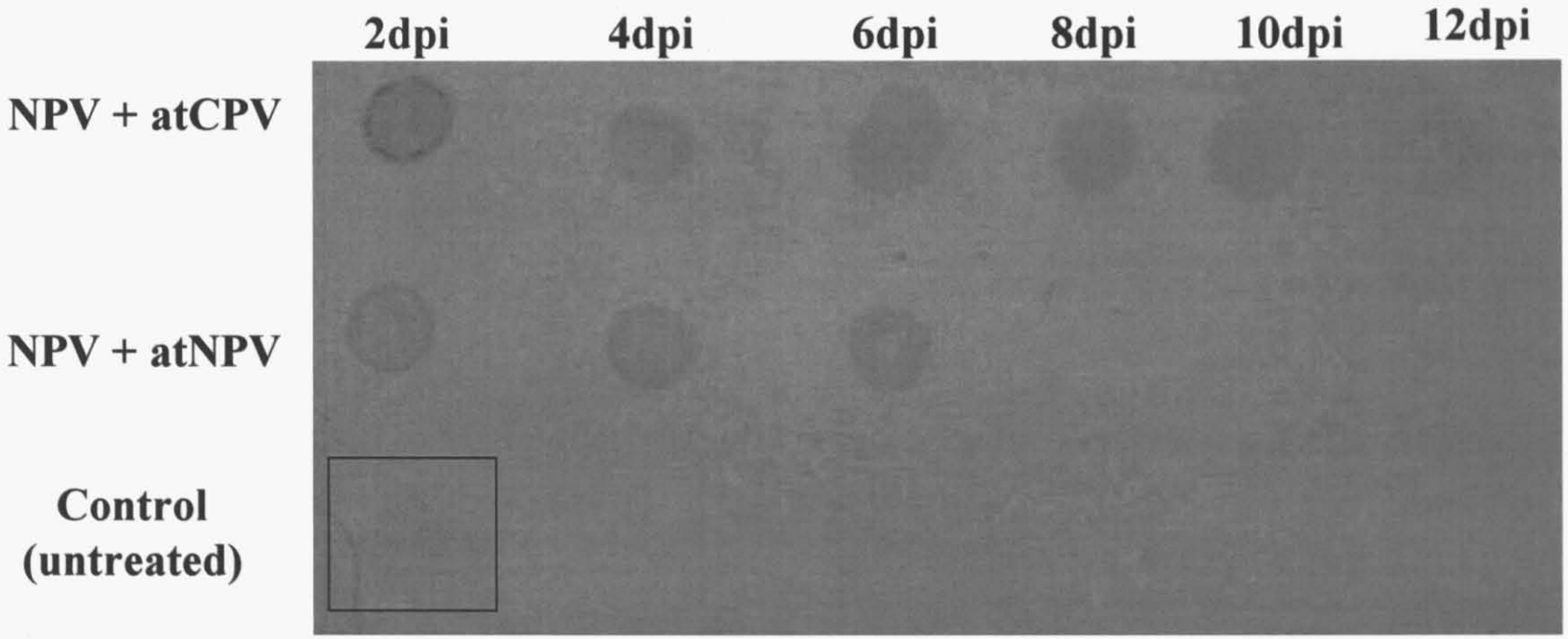

Fig. 2-3 Dot Immuno-Binding Assay to check the antibody remaining in the hemolymph without disrupting its tertiary structure

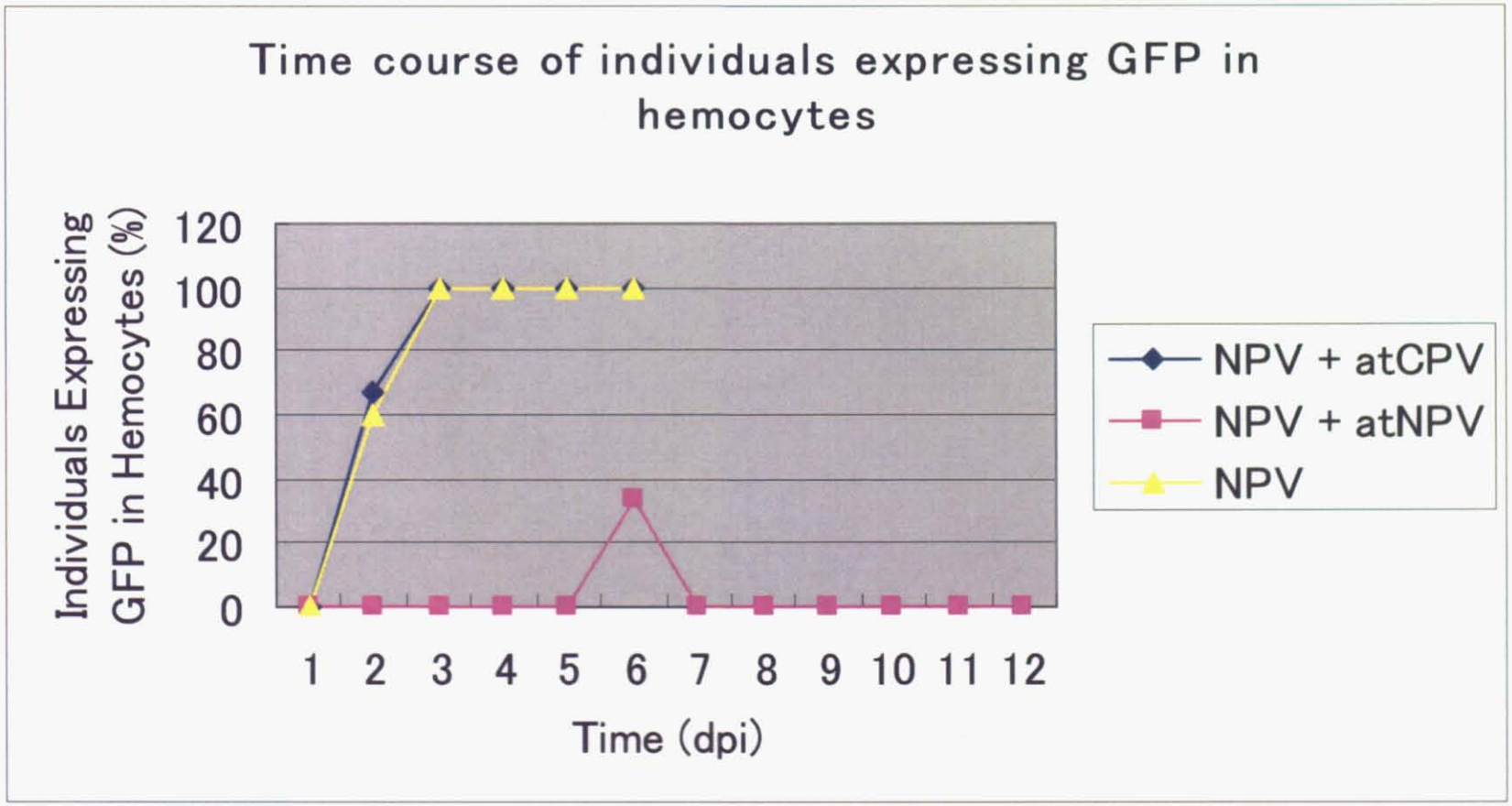

Fig. 2-4 Time course of individuals expressing GFP in hemocytes.
NPV represents the larvae orally infected with GFP recombinant BmNPV. NPV + atCPV or atNPV represent the larvae orally infected with the virus, and treated with anti-CPV or anti-NPV anti-serum at 6hpi. Hemolymph was collected from 3 larvae at each time point, and observed separately under fluorescent microscope.

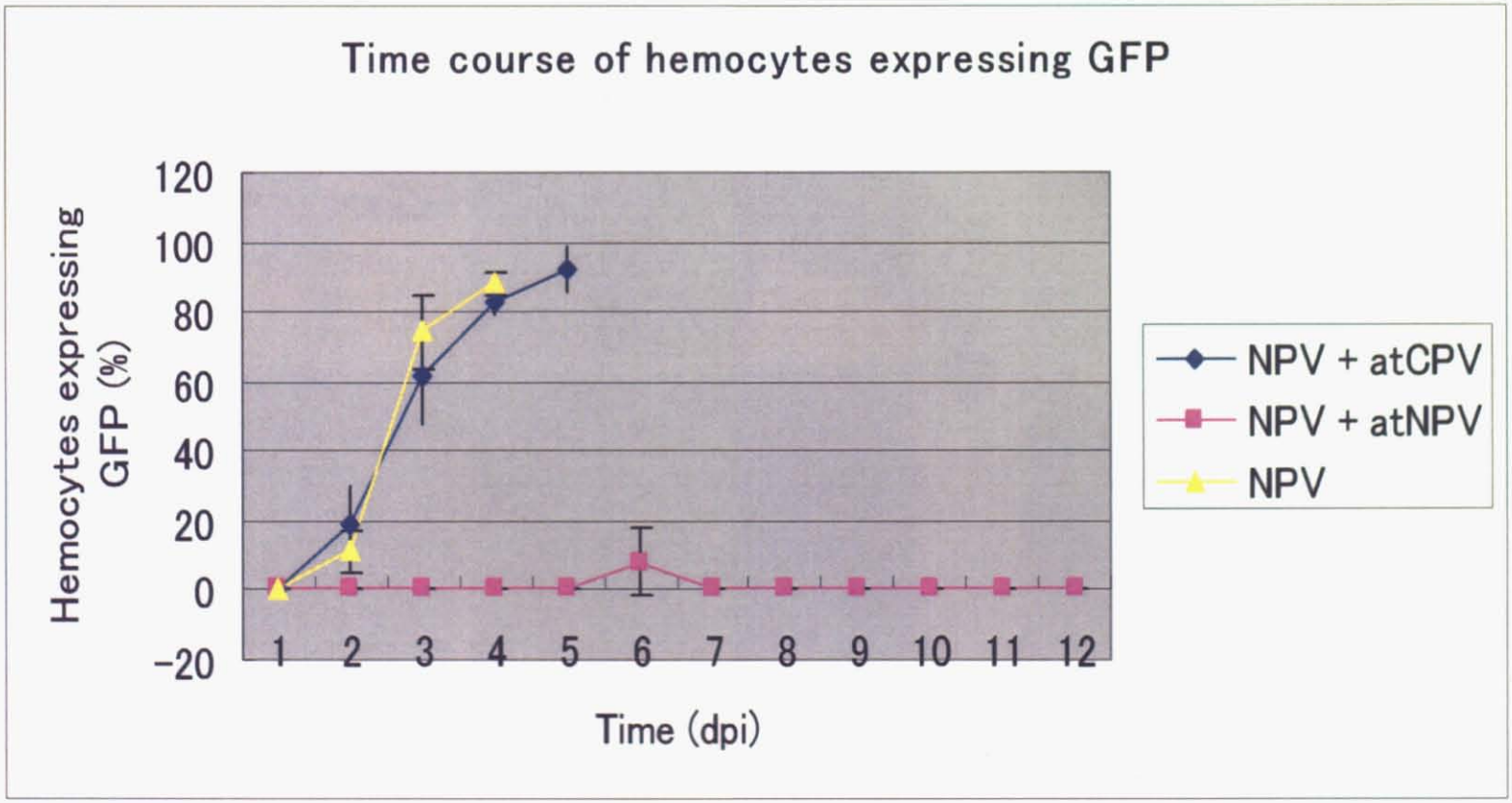

Fig. 2-5 Time course of hemocytes expressing GFP.

NPV represents the larvae orally infected with GFP recombinant BmNPV. NPV + atCPV or atNPV represent the larvae orally infected with the virus, and treated with anti-CPV or anti-NPV anti-serum at 6hpi. Hemolymph was collected from 3 larvae at each time point, and observed separately under fluorescent microscope.

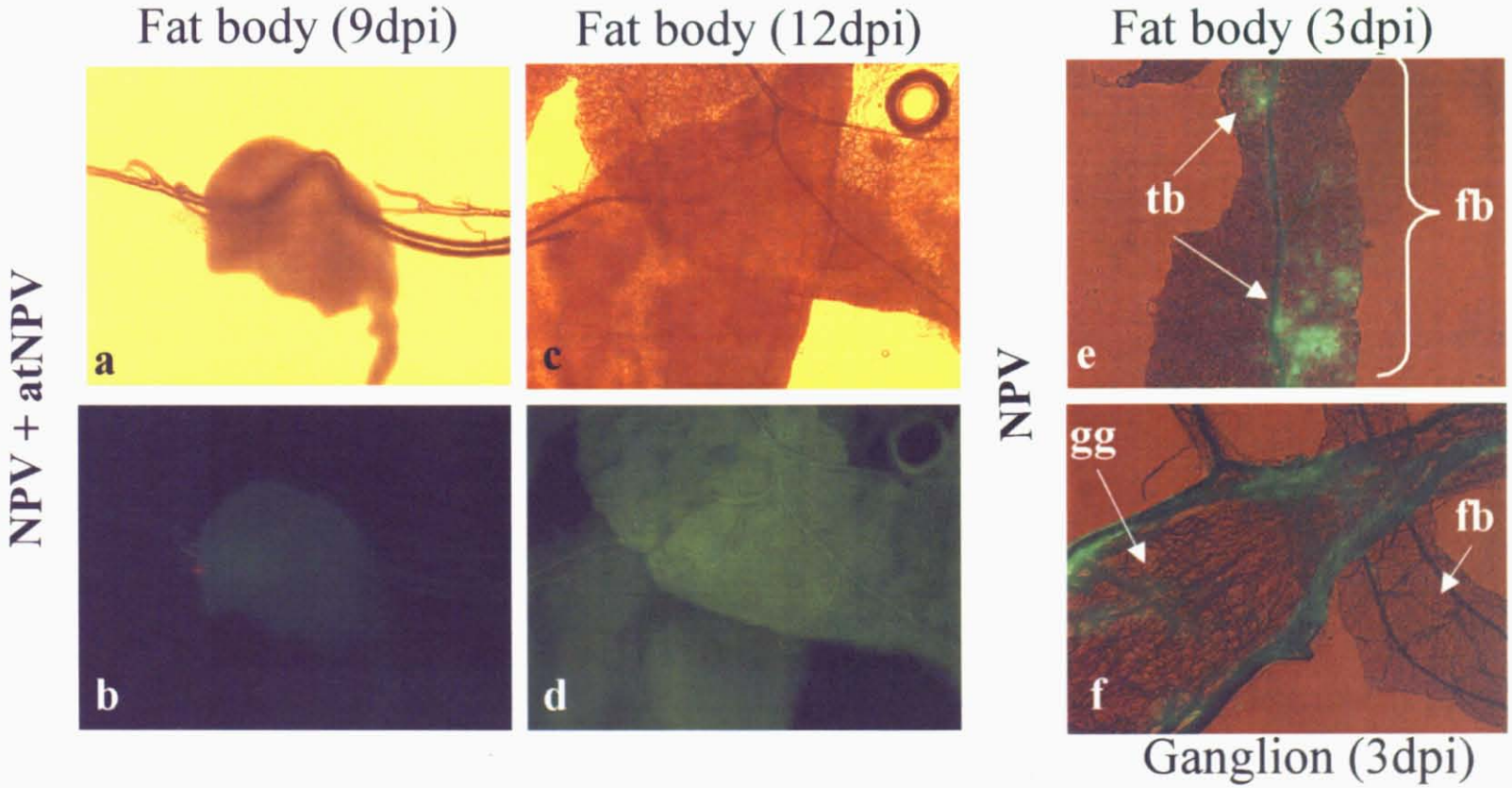

Fig. 2-6 Observation of GFP fluorescence in tissues of *B.mori* infected with recombinant BmNPV and the effect of anti-NPV serum treatment.

a-d: NPV + atNPV; e,f : NPV only; a and c: blight field image; b,d and f: fluorescence image; Note the absence of tracheoblast infection of atNPV treated larvae (b and d), which was seen in the early phase of NPV infection (e and f).

fb: fat body; gg: ganglion; tb: tlacheoblast

Citation:

1. Akai,H, Sato, S. (1973) Ultrastructure of the larval hemocytes of the silkworm, *Bombyx mori L.* (Lepidoptera: Bombycidae). Int J Insect Morphol Embryol 2: 207-231

2. Barret, J. W., Brownwright, A. J., Primavera, M. J., and Palli, S. R. (1998) Studies of the Nucleopolyhedrovirus Infection Process in Insects by Using the Green Fluorescence Protein as a Reporter, Journal of Virology 72: 3377-3382

3. Carton Y, Nappi AJ (1997) *Drosophilia* cellular immunity against parasitoids. Parasitol Today 13:218–227

4. Carton Y, Nappi AJ (2001) Immunogenetic aspects of the cellular immune response of *Drosophilia* against parasitoids. Immunogenetics 52:157–164

5. Engelhard, E. K., Kam-morgan, L. N. W., Washburn, J. O., and Volkman, L. E. (1994) The insect tracheal system: A conduit for the systemic spread of Autographa californica M nuclear polyhedrosis virus. Proc. Natl. Acad. Sci., 91, 3224-3227

6. Granados, R. R. and Williams, K. A. (1986) The biology of Baculoviruses, R.R. Granados and B. A. Federici Eds. (CRC Press, Boca Raton FL, 1986) vol. 1, pp. 89-108

7. Granados, R. R., and Lawler, K. A. (1981) In vivo pathway of Autographa californica

baculovirus invasion and infection. Virology 108, pp297-308

8. Hawks, R.,Niday, E. and Gordon, J. (1982) A dot immunobinding assay for monoclonal and other antibodies, Anal. Biochem., 119, 142-147

9. Hess, R. T. and Falcon, L. A. (1987) J. Invertebr. Pathol. 50, 85-105.

10. Hoffmann JA, Reichhart JM, Hetru C (1996) Innate immunity in higher insects. Curr Opin Immunol 8:8–13

11. S. Katsuma, T. Shimada and M. Kobayashi (2004) Characterization of the baculovirus *Bombyx mori* nucleopolyhedrovirus gene homologous to the mammalian FGF gene family, *Virus Genes* 29, pp. 211–217.

12. Keddie, B. A., Aponte, G. W. and Volkman, L. E. (1989) The pathway of infection of *Autographa californica* Nuclear Polyhedrosis Virus in an insect host, Science, 243,1728-1730

13. Lackie AM (1988) Immune mechanisms in insects. Parasitol Today 4:98–105

14. Lavine MD, Strand MR (2002) Insect hemocytes and their role in immunity. Insect Biochem Mol Biol 32:1295–1309

15. Ling, E., Shirai, K., Kanematsu, R. and Kiguchi, K. (2005) Hemocyte differentiation in the hematopoietic organs of the silkworm, Bombyx mori: prohemocytes have the function of phagocytosis. Cell Tissue Res, 320: 535-543

16. Mori, K. (1970) カイコによる新生物実験 - 生物化学の新展開 - 森 精編. (Sanseido Press, Tokyo, Japan, 1970) pp. 349-352

17. Nagata, M. and Sun P. J. (1998) Multiplication of nucler polyhedro virus in the silkworm larvae during the latter period of the fifth-instar

18. Nittono, Y. (1960) Studies on the blood cells in the silkworm, Bombyx mori, L. Bull Sericult Exp Sta 16: 171-266

19. O' Reilly, D. R., and Miller, L. K. (1989) A baclulovirus blocks insect molting by producing ecdysteroid UDP-glucosyl transferase, Science, 245, pp. 1110-1112

20. Rahman, M. M. and Gopinathan, K. P. (2004) Systemic and in vitro infection process of *Bombyx mori* nucleopolyhedrovirus, Virus Research, 101, pp. 109-118

21. Reddy, J.T. and Locke, M. (1990) The size limited penetration of gold particles through insect basal laminae, J. Insect Physiol. 36, pp. 397-408

22. Tanada, Y. and Hess, R. T. (1991) in Atlas of Invertebrate Viruses, eds. Adams, J. R. and Bonami, J. R. (CRC, Boca Raton, FL), pp. 247-248.

23. Trudeau, D., Washburn, J. O. and Volkman, L. E. (2000) Central Role of Hemocytes in Autographa californica M Nucleopolyhedrovirus Pathogenesis in Heliothis viresces and Helicoverpa zea, Journal of Virology. 75, pp.996-1003.

24. Wago H (1991) Phagocytic recognition in *Bombyx mori*. In: Gupta AP (ed)

Immunology of insects and other arthropods. CRC Press, Boca Raton, pp. 215–235

25. Whitten, J. M. (1972) Comparative Anatomy of The Tracheal System, Annual Review of Entmology. 17, pp. 373-402.

26. Zhou, Q., Krebs, J.F., Snipas, S.J., Price, A., Alnemri, E.S., Tomaselli, K.J. and Salvesen, G.S., 1998. Interaction of the baculovirus anti-apoptotic protein p35 with caspases: specificity, kinetics, and characterization of the caspase/p35 complex. *Biochemistry* 37, pp. 10757–10765.

27. http://www.jica.go.jp/evaluation/before/2002/india_01.html (JICA　事業事前評価表　インド養蚕普及強化計画)