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*J Immunol* published online 6 November 2015
http://www.jimmunol.org/content/early/2015/11/06/jimmunol.1500613

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Protection of Insects against Viral Infection by Apoptosis-Dependent Phagocytosis

Firzan Nainu,* †Yumiko Tanaka, ‡ Akiko Shiratsuchi,* † and Yoshinobu Nakanishi* †

We investigated whether phagocytosis participates in the protection of insects from viral infection using the natural host–virus interaction between Drosophila melanogaster and Drosophila C virus (DCV). Drosophila S2 cells were induced to undergo apoptotic cell death upon DCV infection. However, UV-inactivated virus was unable to cause apoptosis, indicating the need for productive infection for apoptosis induction. S2 cells became susceptible to phagocytosis by hemocyte-derived l(2)mbn cells after viral infection, and the presence of phagocytes in S2 cell cultures reduced viral proliferation. Phagocytosis depended, in part, on caspase activity in S2 cells, as well as the engulfment receptors Draper and integrin β in phagocytes. To validate the in vivo situation, adult flies were abdominally infected with DCV, followed by the analysis of fly death and viral growth. DCV infection killed flies in a dose-responding manner, and the activation of effector caspses was evident, as revealed by the cleavage of a target protein ectopically expressed in flies. Furthermore, hemocytes isolated from infected flies contained DCV-infected cells, and preinjection of latex beads to inhibit the phagocytic activity of hemocytes accelerated fly death after viral infection. Likewise, viral virulence was exaggerated in flies lacking the engulfment receptors, and was accompanied by the augmented proliferation of virus. Finally, phagocytosis of DCV-infected cells in vitro was inhibited by phosphatidylserine-containing liposome, and virus-infected flies died early when a phosphatidylserine-binding protein was ectopically expressed. Collectively, our study demonstrates that the apoptosis-dependent, phosphatidylserine-mediated phagocytosis of virus-infected cells plays an important role in innate immune responses against viral infection in Drosophila. The Journal of Immunology, 2015, 195: 000–000.

Viruses are among the most malicious entities for human beings. As obligate intracellular parasites, they can infect all types of cellular life forms, ranging from simple Archaea and bacteria to more complex hosts, such as animals and plants (1, 2). During productive infection, viruses enter the host cell and hijack its machinery to replicate at the host’s expense. The resulting new virions are released from the infected cell to infect other healthy cells. When infection systemically expands, tissue homeostasis in the host is devastated, often ending with the death of the host organism (3).

To counter this dangerous invader, host organisms are equipped with a variety of self-defense mechanisms. The most efficient one, which is conserved among evolutionarily higher organisms, involves the actions of killer cells, or CTLs, that kill and eliminate virus-infected cells (4, 5). This relies on the activation of relevant immune cells by APCs and, thus, is a part of adaptive immunity. Most viruses possess an RNA genome, and a vast amount of dsRNA is produced in the infected cells during viral replication. The host exploits this by-product to deploy another mechanism, the innate immune response, to fight against invading viruses. A classical mechanism of this category is accomplished by IFN, a cytokine that is induced by dsRNA and creates an antiviral state in host cells (6–8). The third mechanism involves RNA interference (RNAs), by which the viral genome or transcripts are destroyed (9–12). A newly discovered mechanism adopts autophagy for the elimination of intracellular virus (13–15). In addition, there is another mechanism to target virus-infected cells that does not rely on the action of lymphocytes. We reported previously that influenza virus–infected cells are induced to undergo apoptosis (16) and become susceptible to phagocytosis (17, 18). Through this mechanism, virus contained in infected cells is digested by macrophages and neutrophils, culminating in the mitigation of inflammatory damage in mice caused by the infection with influenza virus (17, 19). Despite such a comprehensive defense strategy, host organisms are not always successful in escaping from infectious diseases caused by viral infection, mostly because pathogens have acquired a strategy to avoid immune responses of the host (20, 21).

Insects possess only an innate immune system to fight against microbial infection (22–24). The fundamental mechanism underlying this type of immunity is quite similar between insects and humans (22, 23). Therefore, the fruit fly Drosophila melanogaster has served as a useful animal model to study innate immune responses owing to its powerful genetic system (25, 26). Until now, studies on the antiviral immunity of Drosophila have focused on how the virus entity is eliminated, but how host cells, once infected, become targeted has not been paid much attention. We focused on this issue using Drosophila C virus (DCV), a non-enveloped positive-strand RNA virus assigned to the Dicistroviridae.
PARP::Venus

nation of virus-infected cells) plays a role in antiviral immunity cell line (33, 34). In the current study, we investigated whether upon infection with DCV, which involve RNAi (11, 29, 30), the Toll-Dorsal pathway (31), and the JAK-STAT pathway (32, 33), the existence of several modes of antiviral immunity activated in

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in contrast, a mechanism involving cellular immunity also was reported: DCV itself is phagocytosed by a Drosophila hemocyte
cell line (33, 34). In the current study, we investigated whether another mechanism known in mammals (i.e., phagocytic elimination of virus-infected cells) plays a role in antiviral immunity and found that this is the case.

Materials and Methods

Cell culture and fly maintenance

l(2)mbn cells, a cell line established from larval hemocytes of a tumorous Drosophila mutant, were maintained at 25°C in Schneider’s Drosophila medium (Life Technologies Japan, Tokyo, Japan) containing 10% (v/v) FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Prior to use in an assay for phagocytosis, l(2)mbn cells at ~70–80% confluence were incubated with 20-
hydroxyecdysone (Sigma-Aldrich Japan, Tokyo, Japan) (1 μM) for 48 h. S2 cells, a cell line established from Drosophila embryos, were cultured similarly to l(2)mbn cells. All flies were maintained with standard cornmeal-agar medium at 25°C. The following fly lines were used in this study: w1118 (Bloomington Drosophila Stock Center, Indiana University, Bloomington, IN) as control flies, drpr(5) clone 15 lacking Draper, beta tubulin-α2 (35) lacking integrin βv, beta tubulin-α2 (35) lacking integrin βv (36), UAS-MFGE8 (37), UAS-MFGE8-ΔC2 (37), da-Gal4 (38), UAS-CD8:: PARP; Venus (39), and pan-Gal4 UAS-GFP (40). The original line drpr(5) (41) was backcrossed with w1118 because we experienced difficulty in its maintenance; we obtained a stable line that was operationally named “clone 15”), with no detectable expression of Draper.

Preparation, titration, and UV inactivation of DCV and infection of S2 cells and flies

To prepare a stock of DCV, S2 cells were mixed with DCV and incubated at 25°C for 4 d. The culture media were collected and triturated by the Reed-Muench method (42) to determine a 50% tissue culture–infective dose (TCID50). In brief, S2 cells (2.5 × 105 in 150 μl medium) were seeded in wells of a 96-well culture dish, incubated overnight, inoculated with the DCV-containing medium at 10-fold serial dilutions, and maintained for 7 d. Each culture well was examined and assigned to be positive or negative for viral infection based on the emergence of cell debris, and the virus titer was determined as described previously (43). The virus preparation, at a concentration of 1.6 × 105 TCID50/ml, was kept frozen at −80°C as a stock until use. To inactivate DCV, an original virus stock was exposed to UV light for 5 min three times at room temperature using a 15W lamp emitting 253.7-nm light at a distance of 10 cm. In all of the in vitro experiments, S2 cells were infected with 40 TCID50 of DCV or mock infected and maintained at 25°C until analysis. Infection of adult flies with DCV was carried out according to an established procedure (44) with slight modifications. Briefly, male flies, 3–6 d after eclosion, were anesthetized with CO2 (15–20 flies/vial, three vials in each experiment) and abdominally injected with ~50 nl of the virus preparation (~80,000 TCID50 in the experiments shown in Figs. 5B, 5E–H, and 6, and ~8,000 TCID50 in the experiments shown in Fig. 7B, 7C, and 8) using a glass needle with the aid of a nitrogen gas-operated microinjector. Another batch of flies was similarly injected with PBS as a negative control. These flies were maintained at 25°C until examination. We considered fly death within the first 2 h of injection to be due to injury with the needle and excluded those flies from the analyses. To determine the amount of virus contained in flies, five live flies were placed in a microtube, homogenized using a plastic pestle with Drosophila lysis buffer consisting of 50 mM Tris-HCl (pH 7.5), 0.1% (v/v) Nonidet P-40, 10% (v/v) glycerol, 0.1% (v/v) Triton X-100, 0.15 M NaCl, 5 mM MgCl2, and 1% (v/v) protease inhibitor mixture (Nacalai Tesque, Kyoto, Japan), and centrifuged. The resulting supernatants were subjected to virus titration, as described above.

Western blotting

To detect the capsid proteins of DCV, we used anti-DCV Ab, which was raised by immunizing chickens with DCV and purified (44), or anti-DCV capsid polyprotein Ab (ab92954; Abcam, Cambridge, U.K.), which was raised in rabbits by immunization with a synthetic peptide corresponding to C-terminal residues of the capsid polyprotein and affinity purified. Anti-Drosophila inhibitor of apoptosis protein 1 (DIAP1) antisera was generated by immunizing rabbits with a recombinant DIAP1 protein (45). Anti-Drosophila IL-1 converting enzyme (drlCE) Ab was generated by immunizing rabbits with a synthetic peptide corresponding to residues surrounding Asp230 of drlCE and affinity purified (#13085; Cell Signaling Technology Japan, Tokyo, Japan). Anti-poly-ADP-ribose) polymerase (PARP) Ab was generated by immunizing rabbits with a synthetic peptide corresponding to the caspase cleavage site of human PARP and affinity purified (#9542; Cell Signaling Technology Japan). Anti-Draper, anti-integrin βv, and anti-DmCaBP1 antisera were raised by immunizing rats with recombinant proteins corresponding to aa 881–1200 (intracellular region) of Draper (46), aa 753–799 (intracellular region) of integrin βv (36), and full-length DmCaBP1 (47), respectively. To obtain protein samples for Western blotting, cells and whole flies were lysed with Drosophila lysis buffer and centrifuged. The resulting supernatants were separated by SDS-PAGE and subjected to Western blotting, according to a standard procedure. As the secondary Ab, HRP-conjugated anti-chicken IgY Ab (Jackson Immunoresearch, West Grove, PA), HRP-conjugated anti-rabbit IgG Ab (GE Healthcare Bio-Sciences KK, Tokyo, Japan), and alkaline phosphatase-conjugated anti-rat IgG Ab (KPL, Gaithersburg, MD) were used. Signals were visualized using Western Lightning (PerkinElmer Japan, Yokohama, Kanagawa, Japan) for the HRP-conjugated Ab and the Immun-Star system (Bio-Rad, Tokyo, Japan) for the phosphatase-conjugated Ab.

Assay for phagocytosis in vitro

Hormone-treated l(2)mbn cells (2 × 106 in 1 ml medium) were seeded on a coverslip contained in wells of a 24-well culture dish and maintained in Schneider’s Drosophila medium containing 1% FBS at 25°C for 2 h. They were supplemented with DCV- or mock-infected S2 cells (2 × 105 cells) and incubated until analysis. The cultures were washed with PBS and successively treated with PBS containing 2% (v/v) paraformaldehyde, 0.1% (w/v) glutaraldehyde, and 0.05% Triton X-100, as well as with methanol for fixation. They were washed with PBS containing 0.1% Triton X-100 (PBST), treated with PBST containing 5% (v/v) whole swine serum for blocking, and incubated with PBST containing anti-DCV Ab at 4°C overnight. The samples were washed with PBS, incubated with PBS containing HRP-labeled anti-chicken IgY Ab at room temperature for 1 h, and washed with PBS. They were then subjected to a colorimetric reaction with 3,3'-diaminobenzidine tetrahydrochloride as a substrate, counterstained with hematoxylin, and examined by microscopy. We determined the percentage of l(2)mbn cells that had engulfed DCV-infected S2 cells, as well as the number of DCV-infected S2 cells engulfed by 100 l(2)mbn cells.

Assay for phagocytosis ex vivo

To assess the phagocytosis of DCV-infected flies in vitro, we cytologically analyzed hemocytes isolated from adult flies according to an established procedure (48) with modifications (R. Markus, personal communication). In brief, adult pan-Gal4 UAS-GFP flies specifically expressing GFP in hemocytes (40), infected or not infected with DCV, were anesthetized with CO2, cut at the last segment of the abdomen with a forceps, and injected with PBS at the thorax using a glass capillary. Pressure was gently applied to the capillary so that PBS overflowed as drops together with fly hemolymph. The drops were collected on aminosilane-coated glass slides, and cells contained in hemolymph were examined under a fluorescence microscope for the positivity of GFP signals. The cells were also analyzed for the appearance of nuclei by staining with hematoxylin, as well as for DCV capsid proteins by incubation with anti-DCV capsid polyprotein Ab. GFP* cells that contained viral capsid proteins and two or more nuclei were considered adult hemocytes that had engulfed DCV-infected cells.

Other materials and methods

To examine cell viability and chromatin condensation, DCV-infected S2 cells were incubated with Hoechst 33342, followed by bright-field and fluorescence microscopy, respectively. We considered S2 cells negative for trypan blue staining viable and those intensely stained with Hoechst 33342 to contain condensed chromatin. To inhibit the activity of caspases, benzoxycarboxyl-Val-Ala-Asp-fluoromethylketone (z-VAD-fmk; Peptide Institute, Osaka, Japan) was added to S2 cell cultures at 20 μM 1 h prior to DCV infection. To examine the fragmentation of DNA in situ, cells infected with DCV were labeled with fluoresein isothiocyanate (FITC)-TUNEL assay (ApopTag Peroxidase In Situ Apoptosis Detection Kit; Merck Millipore, Darmstadt, Germany), and signals were visualized in a HRP reaction with 3,3′-tetramethylbenzidine tetrahydrochloride as a substrate

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RNAi knockdown was carried out by incubating l(2)mbn cells \((8 \times 10^6)\) in the presence of dsRNA containing sequences of the mRNA of Draper and integrin βv \((5 \mu g/ml for Draper and 8 \mu g/ml for integrin βv)\), as described previously (46). The sequences used to synthesize dsRNA corresponded to nt 2679–4028 of draper and 265–1247 of betaInt-nu, with the transcription start site numbered 1. To inhibit the phagocytic activity of hemocytes, adult flies were abdominally injected with latex beads \((2 \mu m in diameter; Life Technologies Japan) 6 h prior to the infection with DCV, according to the procedures reported previously (49). Liposome was formed as a suspension in PBS using either phosphatidylcholine only or a combination of phosphatidylcholine and phosphatidylserine at a molar ratio of 7:3, as described previously (50). Phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL).

Data processing and statistical analysis

Results from quantitative analyses are expressed as the mean ± SD of the data from three independent experiments, unless otherwise stated in the figure legends. Other data are representative of at least two independent experiments that yielded similar results. The number of experiments replicated is indicated in the corresponding figure legends. In an assay for fly survival after DCV infection (Figs. 5A, 6A, 6B, 7B), experiments with a single vial were repeated three times to confirm reproducibility and then a final experiment using triplicate vials was carried out. Statistical analyses were performed using the log-rank test (Kaplan–Meier method) for the data from an assay for fly survival or a two-tailed Student t test for all other data. The p values are indicated in the corresponding figures or figure legends; any p values < 0.05 were considered significant.

Results

Induction of apoptosis in Drosophila S2 cells upon infection with DCV

To establish a reliable system for assessing the occurrence of apoptosis-dependent phagocytosis of virus-infected cells, as well as its potential role in providing protection against viral infection in Drosophila, we chose DCV, a nonenveloped RNA virus that is recognized as a natural pathogen of Drosophila. Drosophila embryo-derived S2 cells were incubated with DCV and examined for morphological changes caused by infection with DCV. Microscopic observation showed that DCV-infected S2 cells tended to shrink, giving rise to debris-like small particles (Fig. 1A), indicative of a cytopathic effect of viral infection. We next determined the expression level of viral capsid proteins and the growth of virus. The capsid proteins of DCV appeared as two bands, with \(M_r\) of 38 and 29 kDa, in Western blotting using anti-DCV Ab (Fig. 1B), as reported previously (44). The data showed that the levels of the capsid proteins in S2 cells and the virus released into the culture medium increased in a time-dependent manner (Fig. 1B, 1C), indicating successful infection of S2 cells with DCV.

A previous article reported the induction of apoptosis in Drosophila Line-1 cells upon infection with flock house virus (FHV), another RNA virus belonging to Nodaviridae (51). Thus, we examined possible biochemical changes in S2 cells, which are often observed during apoptosis. DCV-infected cells exhibited reduced viability in a mode inhibitable by the pan-caspase inhibitor z-VAD-fmk (Fig. 2A). The occurrence of chromatin condensation upon infection with DCV was observed, which was also inhibited by the addition of z-VAD-fmk (Fig. 2B). DNA fragmentation, as revealed by a TUNEL assay, became evident in S2 cells postinfection with DCV (Fig. 2C). We then determined the levels of apoptosis-related proteins by Western blotting in the lysates of S2 cells that were infected or mock-infected with DCV. The

![FIGURE 1.](http://www.jimmunol.org/)

Cytopathic effect and propagation of DCV in Drosophila S2 cells. (A) Morphological changes in DCV-infected cells. S2 cells were subjected to microscopic analysis at the indicated time points postinfection with DCV. The lower panels are higher-power views of the dashed boxes in the upper panels. The data from one of two independent experiments that yielded similar results are presented. Scale bars, 100 μm (upper panels), 50 μm (lower panels). (B) Expression of the capsid proteins of DCV during a course of infection. S2 cells were lysed at the indicated time points and analyzed by Western blotting with anti-DCV Ab. A portion of the blotted membrane after a chemiluminescence reaction is shown. The arrowheads denote positive signals. The data from one of two independent experiments that yielded similar results are presented. (C) Propagation of DCV during a course of infection. The culture medium was collected at the indicated time points and analyzed for the virus titer. Representative data of two independent experiments that gave similar results are shown.
FIGURE 2. Apoptotic changes in DCV-infected S2 cells. (A) Decrease in the viability of S2 cells during a course of infection. S2 cells collected at the indicated time points were subjected to a dye exclusion test with trypan blue (left panel). The data are representative of four independent experiments that yielded similar results. S2 cells were infected with DCV for 3 d in the presence or absence of z-V AD-fmk and analyzed for viability (right panel). Mean ± SD of the data obtained from three independent experiments are shown. (B) Occurrence of chromatin condensation in S2 cells during a course of infection. S2 cells infected with DCV for 2 d were subjected to staining with the DNA-binding fluorochrome Hoechst 33342 and examined under a fluorescence/phase-contrast microscope (left panel). Representative images of S2 cells with and without chromatin condensation are presented as fluorescence (upper panels) and phase-contrast (lower panels) views of the same microscopic fields. Scale bars, 5 μm. S2 cells were collected at the indicated time points of infection and analyzed for the occurrence of chromatin condensation (middle panel). The data are representative of four independent experiments that yielded similar results. S2 cells were infected with DCV for 3 d in the presence or absence of z-VAD-fmk and analyzed for condensed chromatin (right panel). Mean ± SD of the data obtained from three independent experiments are shown. (C) DNA fragmentation in S2 cells postinfection with DCV. S2 cells were infected with DCV or mock infected for 3 d, and whole-cell lysates were subjected to Western blotting analysis using the indicated Ab. Portions of the blotted membranes after a chemiluminescence reaction are shown. Lysates prepared from the same number of S2 cells were analyzed with and without DCV infection. Note that the anti-DCV capsid polyprotein Ab was used to detect capsid proteins. The open and filled arrowheads (second panel) point to intact and cleaved (Figure legend continues)
activation of drICE, an effector caspase of *Drosophila*, by partial cleavage was seen after viral infection (second panel, Fig. 2D). Apoptosis caused by FHV infection is accompanied by a decrease in DIAPI (51). DIAPI was detectable as a protein band of 57 kDa in the lysates of uninfected S2 cells, as originally reported (45), but we observed a decrease in this caspase inhibitor after DCV infection (third panel, Fig. 2D). The reduction in DIAPI expression was unlikely to have been due to the general inhibition of host protein synthesis, which was observed in cells infected with FHV (51), because the level of DmCaBP1, a 51-kDa endoplasmic reticulum protein (47) analyzed as an internal control, did not change significantly after viral infection (fourth panel, Fig. 2D). These data collectively suggested that DCV infection induces apoptotic cell death in S2 cells through the activation of caspases after the loss of DIAPI. We next examined whether growth-defective virus is capable of inducing apoptosis. To do so, DCV was exposed to UV light and used to infect S2 cells. UV-treated virus did not grow effectively in S2 cells (first panel, Fig. 2D, left panel, Fig. 2E), indicating successful inactivation of virus. We found that TUNEL positivity (right panel, Fig. 2E) and drICE cleavage (Fig. 2D, second panel) were not observed in S2 cells infected with inactive DCV. These results indicated that productive infection is necessary for this virus to induce caspase-mediated apoptosis in host cells.

**Engulfment of DCV-infected cells and inhibition of virus proliferation by *Drosophila* phagocytes**

In general, cells undergoing apoptosis become susceptible to phagocytosis (52–54). Thus, we tested whether DCV-infected, apoptotic S2 cells are engulfed by *Drosophila* phagocytes in vitro. For this purpose, we used l(2)mbn cells, a *Drosophila* larval hemocyte-derived cell line that was shown to engulf apoptotic cells when activated by incubation with the molting hormone 20-hydroxyecdysone (46). Spherical S2 cells were morphologically distinguishable from hormone-treated l(2)mbn cells that showed a macrophage-like flattened shape (middle panel, Fig. 3A). DCV-infected S2 cells and hormone-treated l(2)mbn cells were cocultivated and subjected to immunocytochemistry with anti-DCV Ab to detect viral infection, followed by counterstaining with hematoxylin for visualization of the nucleus. S2 cells before and postinfection were stained blue and in a combination of blue and brown colors, respectively (left panel, Fig. 3A). We found that some l(2)mbn cells included brownish particles (right panel, Fig. 3A), and we considered them phagocytes that had

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**FIGURE 3.** Phagocytosis of DCV-infected S2 cells and inhibition of viral growth by *Drosophila* hemocyte cell line. (A) Microscopic observation of phagocytosis. S2 cells infected with DCV were incubated with 20-hydroxyecdysone-treated l(2)mbn cells, subjected to immunocytochemistry with anti-DCV Ab and counterstaining with hematoxylin, and examined by microscopy. Cells labeled a–d are uninfected S2 cells, DCV-infected S2 cells, l(2)mbn cells, and l(2)mbn cells that engulfed DCV-infected S2 cells, respectively. The open and filled arrowheads point to a DCV+ S2 cell contained in an l(2)mbn cell, and the nucleus of a l(2)mbn cell, respectively. Scale bars, 10 μm. (B) Time-course analysis of phagocytosis. S2 cells infected with DCV were cocultivated with hormone-treated l(2)mbn cells for the indicated periods and analyzed for the percentage of l(2)mbn cells that had accomplished phagocytosis (left panel), as well as the number of S2 cells that had been engulfed by 100 l(2)mbn cells (right panel). Mean ± SD of the data obtained from three independent experiments are presented. (C) Virus propagation in the presence of phagocytes. S2 cells infected with DCV for 18 h were further cultured in the presence and absence of hormone-treated l(2)mbn cells, and the amount of virus existing in the culture medium was determined at the indicated time points. Mean ± SD of the data obtained from three independent experiments are presented.
engulfed DCV-infected S2 cells. The extent of phagocytosis was evaluated based on the level of l(2)mbn cells containing DCV + S2 cells (left panel, Fig. 3B), as well as the number of target cells engulfed by a given number of l(2)mbn cells (right panel, Fig. 3B). We observed time-dependent increases in phagocytosis of DCV-infected cells for both criteria. Furthermore, the presence of l(2)mbn cells in cultures reduced the level of virus released from S2 cells into the culture medium (Fig. 3C), suggesting that the engulfment of DCV-infected S2 cells culminated in the digestion of virus in l(2)mbn cells.

**Apoptosis-dependent, Draper- and integrin βv–mediated phagocytosis of DCV-infected S2 cells**

To determine whether the phagocytosis of DCV-infected S2 cells by l(2)mbn cells is dependent on apoptosis in the target cells, we conducted an assay for phagocytosis using S2 cells that had been infected with DCV in the presence of z-V-AD-fmk. We found that drug-treated S2 cells showed reduced susceptibility to phagocytosis by l(2)mbn cells in terms of both the percentage of engulfing phagocytes and the number of engulfed cells (Fig. 4A), indicating that the phagocytosis depends, at least in part, on the occurrence of apoptosis in DCV-infected cells. Draper (46) and integrin αPS3-βv (36, 55) are major engulfment receptors that are responsible for the activation of signaling pathways leading to the phagocytosis of apoptotic cells by hemocytes of *Drosophila*. Thus, we next investigated whether l(2)mbn cell phagocytosis of DCV-infected S2 cells requires the action of those receptors. For this purpose, hormone-treated l(2)mbn cells were incubated with dsRNA that possessed sequences corresponding to parts of the mRNA of Draper and integrin βv. Western blotting showed that l(2)mbn cells were almost depleted of Draper, integrin βv, or both, whereas the level of DmCaBP1 analyzed as an internal control was unaffected (Fig. 4B).

**FIGURE 4.** Apoptosis-dependent, Draper- and integrin βv–mediated phagocytosis of DCV-infected S2 cells. (A) Phagocytosis of DCV-infected S2 cells in the presence of caspase inhibitor. S2 cells were infected with DCV for 3 d in the presence or absence of z-V-AD-fmk and used as target cells in an assay for phagocytosis by 20-hydroxyecdysone-treated l(2)mbn cells. Mean ± SD of the data obtained from three independent experiments are presented. (B) RNAi knockdown of engulfment receptors in phagocytes. Hormone-treated l(2)mbn cells were incubated for 3 d with dsRNA possessing sequences corresponding to Draper or integrin βv mRNA and subjected to Western blotting for the levels of Draper, integrin βv, and DmCaBP1 as an internal control. Portions of the blotted membranes after a chemiluminescence reaction are shown. The positions of M, markers are indicated to the left of each panel. The data are representative of three independent experiments that yielded similar results. (C) Phagocytosis of DCV-infected S2 cells by engulfment receptor-depleted l(2)mbn cells. S2 cells were infected with DCV for 3 d and subjected to an assay for phagocytosis using dsRNA-treated l(2)mbn cells as phagocytes. Mean ± SD of the data obtained from three independent experiments are presented.

Those RNAi-induced l(2)mbn cells were used in an assay for phagocytosis, with DCV-infected S2 cells as targets (Fig. 4C). The reduction in the level of Draper or integrin βv resulted in an ∼60% decrease in the level of phagocytosis. Moreover, the phagocytosis level declined further when the expression of Draper and integrin βv was inhibited simultaneously. These results demonstrated that Draper and integrin βv, major engulfment receptors responsible for the phagocytic elimination of apoptotic cells in *Drosophila*, were independently involved in the phagocytosis of DCV-infected cells. We concluded that the phagocytosis of DCV-infected S2 cells by l(2)mbn cells occurred in a manner dependent on apoptosis in target cells and mediated by the engulfment receptors Draper and integrin αPS3-βv of phagocytes.

**Apoptosis and hemocyte phagocytosis of DCV-infected cells in *Drosophila***

We carried out in vivo experiments to determine the role of phagocytosis in the protection of *Drosophila* from DCV infection. To establish an infection model, adult flies were injected abdominally with DCV at various doses, and the rate of death was observed. We found that the higher the dose of virus used for infection, the earlier the flies died (Fig. 5A), indicating successful infection and a pathogenic effect of DCV in adult flies. We next examined the occurrence of apoptosis in DCV-infected flies by determining the cleavage of PARP, a target of effector caspases. For this purpose, we analyzed flies that express a portion of human PARP, including the cleavage site for caspases fused to the extracellular and transmembrane domain of murine CD8 at the N terminus, and Venus, a bright variant of YFP, at the C terminus (CD8-PARP-Venus) postinfection with DCV. When whole-fly lysates were analyzed by Western blotting with anti-PARP Ab, the lysates of infected flies showed an increase in the level of a signal...
corresponding to the cleaved CD8-PARP-Venus protein (Fig. 5B). As seen in the experiments using S2 cells, such an apoptosis-associated change was mitigated when flies were infected with UV-inactivated virus (Fig. 5B). These results indicated that infection with DCV induces caspase-mediated apoptosis in adult flies.

We then tried to obtain evidence for the phagocytosis of DCV-infected cells in flies. Hemolymph was collected from adult flies that express GFP in hemocytes and analyzed for the presence of hemocytes that contain cells infected with DCV. When cells in the hemolymph were examined by fluorescence microscopy, 

90% were positive for GFP (Fig. 5C, 5C'), indicating that most cells obtained in this way were hemocytes. Hemocytes isolated from uninfected flies showed a macrophage-like flattened shape, as seen for 20-hydroxyecdysone–treated l(2)mbn cells (Fig. 3A), and possessed small nuclei, as revealed by hematoxylin staining (Fig. 5D). In contrast, hemocytes of DCV-infected flies included those containing two or more nuclei: one nucleus was the size of the hemocyte nucleus, whereas the others were larger (Fig. 5E).

When those hemocytes were subjected to immunostaining using anti-DCV capsid polyprotein Ab, a large particle within a hemocyte showed a positive signal (Fig. 5F) and a part of which also stained with hematoxylin (Fig. 5G). These results suggested that larger hematoxylin + signals in hemocytes are from engulfed DCV-infected cells. We considered adult hemocytes containing multiple nuclei and viral capsid proteins to be those that had phagocytosed DCV-infected cells; such cells accounted for ∼10% of total hemocytes obtained from virus-infected flies and were rarely found in hemocytes of uninfected flies (Fig. 5H).

**Protection of Drosophila against DCV infection by Draper- and integrin βv–mediated phagocytosis**

We next examined the role of phagocytosis in antiviral mechanisms of Drosophila. Adult flies were abdominally injected with undigestible latex beads to inhibit the phagocytic activity of hemocytes prior to infection with DCV. Bead-injected flies died earlier than did those injected with PBS only after viral infection (Fig. 6A),
suggesting a protective role for phagocytosis against viral infection. We next compared the rate of fly death for flies lacking engulfment receptors and those expressing them normally. The fly lines with a null mutation in *drpr* (coding for Draper) or *betaInt-nu* (coding for integrin βν) died at similar rates and earlier than control flies (Fig. 6B), indicating almost equal contributions of Draper and integrin βν to the survival of flies postinfection with DCV. Furthermore, flies lacking both Draper and integrin βν became even more vulnerable to DCV infection (Fig. 6B), suggesting independent actions of these two engulfment receptors, as observed in the experiment examining phagocytosis in vitro (Fig. 4C). The growth of DCV was then analyzed by determining the amount of virus existing in the lysates of infected flies. We found that the virus titer in the lysates increased with prolonged time of infection (Fig. 6C), indicating DCV propagation in adult flies. When the effect of the loss of the engulfment receptors was examined, almost equal increases in the levels of virus production in flies lacking either Draper or integrin βν were observed (Fig. 6C). In line with the data for fly survival, the virus titer in flies possessing mutations in both *drpr* and *betaInt-nu* was higher than that in flies with single mutations (Fig. 6C). Collectively, these data indicated that the Draper- and integrin βν-mediated phagocytosis of DCV-infected cells helps flies to resist DCV infection.

**FIGURE 6.** Antiviral role for phagocytosis in *Drosophila*. (A) Early death of DCV-infected flies by preinjection of latex beads. Adult flies (w1118) that had been injected with latex beads (Pre-bead) or PBS alone (Pre-PBS) for 6 h were infected abdominally with DCV, and the percentage of live flies was determined at the indicated time points. Average values of the data from three-vial experiments are presented. *p* < 0.0001, Pre-PBS versus Pre-bead (with DCV infection), log-rank test. (B) Pathogenic effect of DCV on flies lacking engulfment receptors. The indicated lines of flies were injected with DCV, and the rate of fly death was determined at the indicated time points. Average values of the data from three-vial experiments are presented. *p* < 0.0001, w1118 versus betaInt-nu2, *p* < 0.0001, w1118 versus drpr5, *p* < 0.0001, w1118 versus betaInt-nu2; drpr5, *p* = 0.2412, betaInt-nu2 versus drpr5, *p* < 0.0001, betaInt-nu2 versus betaInt-nu2; drpr5, *p* < 0.0001, drpr5 versus betaInt-nu2; drpr5 (all with DCV infection), log-rank test. (C) Virus propagation in flies lacking engulfment receptors. The indicated lines of flies were infected with DCV. Lysates of whole flies were prepared at the indicated time points and analyzed for the virus titer. Mean ± SD of the data obtained from three independent experiments are presented. The *p* values within the bars refer to comparison with w1118 flies, whereas those shown above the bars refer to comparison with betaInt-nu2; drpr5 flies. betaInt-nu2, a null-mutation allele of the gene coding for integrin βν; drpr5, a null-mutation allele of the gene coding for Draper.

**Participation of phosphatidylserine in the phagocytosis of DCV-infected cells**

Cells undergoing apoptosis are recognized by phagocytic cells through molecular interaction between markers for phagocytosis presented at the surface of target cells during apoptosis and engulfment receptors of phagocytes (52–54). The glycerophospholipid phosphatidylserine is the most comprehensive marker for phagocytosis among different cell types and species (56–58). Thus, we tested whether this phospholipid serves as a marker for phagocytosis in DCV-infected cells. Liposome containing phosphatidylserine, which was used successfully to inhibit phosphatidylserine-mediated phagocytosis in vitro (59), was included in a phagocytosis reaction using DCV-infected S2 cells and hormone-treated l(2)mbn cells. We found that the presence of phosphatidylserine-containing liposome, but not control phosphatidylcholine-only liposome, reduced phagocytosis to half of the original level (Fig. 7A), indicating the involvement of phosphatidylserine. We then tested the role of phosphatidylserine in vivo using a fly line that systemically expresses milk fat globule–epidermal growth factor 8 (MFG-E8), a mammalian phosphatidylserine-binding protein (60), using the GAL4-UAS system (61). The possession of both UAS and Gal4 transgenes for the systemic expression of MFG-E8 brought about earlier death of DCV-infected flies, whereas this was not the case when flies possessed only one transgene or expressed MFG-E8-ΔC2, which lacks a domain responsible for binding to phosphatidylserine (61) (Fig. 7B). Coinciding with these data, DCV grew more efficiently in flies that expressed MFG-E8 compared with control flies (Fig. 7C), as observed using flies that lacked engulfment receptors (Fig. 6C). These results suggested that the presence of MFG-E8, a phosphatidylserine-binding protein, abrogated the phagocytic elimination of DCV-infected cells. Taking these findings together, the phagocytosis of DCV-infected cells in *Drosophila* requires, at least in part, the exposure of phosphatidylserine in the target cells.

**Exaggeration of morphological change in DCV-infected flies by loss of engulfment receptors**

Recent studies reported changes in the morphology of flies (i.e., abdominal swelling and crop enlargement) upon infection with DCV (31, 62). We wondered whether such morphological changes are due to the expansion of infection. To assess this, we carried out infection experiments using flies lacking engulfment receptors and histologically analyzed them for the size of abdomen and crop. Swollen abdomen and enlarged crop were evident...
in flies, either controls or mutants, postinfection with DCV (left panels, Fig. 8A, 8B), as reported previously. The percentage of flies with such changes increased when they lacked both Draper and integrin βn (right panels, Fig. 8A, 8B). These results suggested that the expansion of infection due to a loss of the Draper- and integrin βn-mediated phagocytic elimination of DCV-infected

FIGURE 7. Involvement of phosphatidylserine in the elimination of DCV. (A) Phagocytosis of DCV-infected S2 cells by l(2)mbn cells in the presence of liposome. S2 cells infected with DCV for 3 d were subjected to an assay for phagocytosis using hormone-treated l(2)mbn cells as phagocytes in the presence and absence of liposome (1 mM). Mean ± SD of the data obtained from three independent experiments are presented. (B) Pathogenic effect of DCV on flies expressing phosphatidylserine-binding MFG-E8. Flies containing the indicated transgenes were injected with DCV, and the rate of fly death was determined at the indicated time points. Average values of the data from three-vial experiments are presented. p = 0.0006, UAS-MFGE8 versus UAS-MFGE8; da-Gal4, p = 0.0002, da-Gal4 versus UAS-MFGE8; da-Gal4, p = 0.6796, UAS-MFGE8ΔC2 versus UAS-MFGE8ΔC2; da-Gal4, p = 0.9526, da-Gal4 versus UAS-MFGE8ΔC2; da-Gal4, p = 0.0002 for UAS-MFGE8; da-Gal4 versus UAS-MFGE8ΔC2; da-Gal4 (all with DCV infection), log-rank test. (C) Virus propagation in flies expressing phosphatidylserine-binding MFG-E8. Flies containing the indicated transgenes were injected with DCV. Lysates of whole flies were prepared at the indicated time points and analyzed for the virus titer. Mean ± SD of the data obtained from three independent experiments are presented. PC, phosphatidylcholine-only liposome; PS, phosphatidylserine-containing liposome.

FIGURE 8. Enhancement of morphological change in DCV-infected flies after loss of engulfment receptors. (A) Abdominal swelling in adult flies after DCV infection. Control (w1118) and Draper- and integrin βn-lacking (betaInt-nu2; drpr16) flies were infected for 8 h after abdominal injection of DCV. They were photographed (left panels), and the percentage of flies with a swollen abdomen was calculated (right panel, n = 20–25). The arrowheads denote the abdomen of the flies. Mean ± SD of the data obtained from three independent experiments are presented. (B) Crop enlargement in adult flies after DCV infection. Control (w1118) and Draper- and integrin βn-lacking (betaInt-nu2; drpr16) flies were infected for 8 h after abdominal injection of DCV. The flies were dissected, and the digestive tract was isolated and photographed (left panels). The arrowheads and arrows point to the crop and the cardia, respectively. Percentage of flies with an enlarged crop (right panel, n = 20–25). Results are the mean ± SD of the data obtained from three independent experiments.

cells (Fig. 6C) brought about an increased rate of morphological changes in the abdomen and crop of Drosophila. Taking these findings together, the phagocytic removal of infected cells plays an important role in the protection of flies from pathological damage caused by infection with DCV.

**Discussion**

In the current study, we demonstrated a role for the phagocytosis of virus-infected cells in the antiviral defense in Drosophila, where the mechanisms relying on RNAi, the Toll-Dorsal pathway, the JAK-STAT pathway, and the phagocytosis of virus itself were emphasized. This indicates the existence of a mechanism in insects whereby invading viruses are eliminated together with host cells rather than the virus entity. Such a mechanism was reported for mammals: influenza virus–infected cells are phagocytosed by macrophages and neutrophils, resulting in the mitigation of pathological damage in the mouse (19). Therefore, it is likely that the phagocytosis of virus-infected cells is an antiviral strategy common among species from insects to mammals. We also claim that this mechanism, evident in insects equipped with only innate immunity, is categorized as an innate immune response. Target specificity in this reaction is due to the occurrence of apoptosis in cells infected with virus. In general, cells induced to undergo apoptosis become susceptible to phagocytosis by surface presentation of a molecule(s) that is recognized by an engulfment receptor of phagocytes (51–53). DCV infection was found to induce caspase-mediated apoptosis in Drosophila S2 cells and was accompanied by several biochemical changes often observed in mammalian cells undergoing apoptosis. Also, the activation of caspases was seen in flies upon infection with DCV. It was appreciated that many types of mammalian virus cause apoptotic cell death in host cells (63). There is another example of an insect virus (i.e., FHV) that induces apoptosis in host cells (51); we can now add DCV to the list. Thus, it is probable that virus infection–triggered apoptosis in host cells is shared by many metazoan species, although it remains to be clarified whether the underlying mechanism is the same. Collectively, we maintain that the apoptosis–dependent phagocytic removal of virus-infected cells is an evolutionarily conserved antiviral strategy of host organisms.

With regard to the molecular basis for the recognition of DCV-infected cells by Drosophila phagocytes, data from our in vitro and in vivo experiments suggested the participation of the glycerophospholipid phosphatidylserine, the best-characterized marker for phagocytosis or eat-me signal (56–58). In contrast, biochemical and genetic studies indicated the involvement of Draper and integrin βv, major engulfment receptors of Drosophila hemocytes (36, 46). We previously identified the endoplasmic reticulum protein Pretaporter (64) and phosphatidylserine (37) as ligands for Draper, whereas it remains to be shown which molecule(s) serves as a ligand for integrin βv, which functions as a heterodimer with αPS3 (55). Therefore, it is reasonable to speculate that Drosophila phagocytes use Draper to recognize phosphatidylserine exposed at the surface of DCV-infected cells. An unknown molecule(s) other than phosphatidylserine likely serves as a ligand for integrin αPS3-βv, because Draper and integrin αPS3-βv seemed to participate independently in the phagocytosis of DCV-infected cells. It is also necessary to resolve how DCV-infected cells undergo apoptosis. The mechanism of apoptosis in Drosophila cells is somewhat different from that in mammalian cells: the initiator caspase DRONC resides in cells in its active form, but its activity is kept dormant by forming a complex with the caspase inhibitor DIAP1 (65). DRONC becomes functional and able to cleave and activate the effector caspases drICE and Dcp-1 when liberated from DIAP1 through the actions of proapoptotic proteins, including Reaper, Hid, and Grim, concomitant with the subsequent degradation of DIAP1 (65). Therefore, a decrease in the level of DIAP1 is an indication of the onset of apoptosis in Drosophila cells. In fact, Settles and Friesen (51) claimed that a decreased level of DIAP1 is solely responsible for the induction of apoptosis in Drosophila Line-1 cells upon infection with FHV. We made a similar observation: DIAP1 decreased in S2 cells after DCV infection. The same research group recently reported another mechanism for the degradation of DIAP1 that is independent of Reaper, Hid, and Grim (66). Therefore, it is important to elucidate the mechanism by which the productive infection of DCV brings about a reduction in the level of this caspase inhibitor for the induction of apoptosis in Drosophila cells.

Our data indicate that the size of viral burden is closely related to the mortality of flies. However, it remains to be determined how flies succumb to DCV infection. DCV infects a variety of tissues and organs in Drosophila, including the fat body, periovular sheath, trachea, muscle, and digestive tract (31, 44, 62, 67, 68). A recent report argued that alteration in a variety of physiological and metabolic phenomena is related to the death of DCV-infected flies (69). We also noticed that DCV-infected adult flies showed retarded migration, but it is unclear whether this phenomenon has anything to do with fly mortality. Another report reasoned that a defect in the uptake of nutrients from the digestive tract is a direct cause of fly death postinfection of the intestine with DCV (62). It is necessary to clarify how infection with virus causes lethal damage in host organisms to develop an effective remedy to prevent and cure viral diseases. The model system we adopted in this study using DCV and Drosophila is suitable for such research because the availability of robust genetics and a huge number of specimens should help to identify the underlying mechanism and the genes involved.

**Acknowledgments**

We thank Dr. Sara Cherry (University of Pennsylvania, Philadelphia, PA) for anti-DCV Ab, Dr. Masayuki Miura (University of Tokyo, Tokyo, Japan) for anti-DIAP1 antiserum and suggestions about an assay for apoptosis in flies, Dr. Takayuki Kuraishi (Tohoku University, Sendai, Miyagi, Japan) for virus stock and suggestions about the infection experiments, Dr. Yasushi Hiromi (National Institute of Genetics, Mishima, Shizuoka, Japan) for the fly line UAS-CD8::Parp;Venus, Dr. Röbert Márkás (University of Nottingham, Nottingham, U.K.) and Dr. István Andó (Biological Research Center of the Hungarian Academy of Sciences, Szeged, Hungary) for suggestions about the preparation of adult hemocytes, Dr. Tsukasa Matsuno (Kanazawa University) for help with UV inactivation of virus, Chieko Nishi (Kanazawa University) for the fly line Dpsk1-1 clone 15, Dr. Kaz Nagaoa (Hirosaki University, Hirosaki, Aomori, Japan) for help with the immunoochemical experiments, and the Bloomington Drosophila Stock Center for fly lines.

**Disclosures**

The authors have no financial conflicts of interest.

**References**

vation of a transcription factor complex containing IRF-3 and CBFβ/p300. EMBO J. 17: 1087–1095.