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Silkworm Hemolymph Inhibits Baculovirus-Induced Insect Cell Apoptosis

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The effect of silkworm hemolymph on baculovirus-induced insect cell apoptosis was investigated. The addition of silkworm hemolymph into the culture medium either before or during the baculovirus infection increased the host cell longevity; however, its addition after the infection was less effective. This can be explained by the higher transfer rate of silkworm hemolymph which is caused by endocytosis during the virus internalization step. The delayed cell death due to silkworm hemolymph was not caused by an inhibition of the virus attachment and internalization steps. The apoptosis was analyzed using DNA fragmentation and TUNEL assays, and the resulting data confirm that silkworm hemolymph inhibits baculovirus-induced insect cell apoptosis.

The insect cell-baculovirus system has received rapid and wide acceptance as an alternative to classical bacterial or yeast systems for the production of proteins from recombinant DNA. This system offers several advantages, including high expression owing to a strong polyhedrin promoter, the production of functionally and immunogenetically active recombinant proteins due to proper post-translational modifications, and the nonpathogenicity of the baculovirus to vertebrates and plants (1).

The infection of an insect cell with a baculovirus induces apoptosis known as active or programmed cell death (2). *Spodoptera frugiperda* and the *Autographa californica* nuclear polyhedrosis virus (AcNPV) have been widely used as the model insect cell and baculovirus, respectively, for studying the baculovirus regulation of host apoptosis. A specific gene product, P35, which prevents an apoptotic response, was identified in AcNPV and the silkworm baculovirus *Bombyx mori* nuclear polyhedrosis virus (BmNPV) (2, 3). Various other genes are also known to regulate apoptosis in the cells of an mammalian immune system (4–6). Baculoviruses also carry genes which produce inhibitors of apoptosis (IAPs). The IAP family was first identified by their ability to substitute for P35 in blocking baculovirus-induced apoptosis (7, 8), and then expanded when IAP homologues were found in a diversity of insect and mammal genomes (9, 10).

Originally, insect hemolymph was used as an insect cell culture medium. Thereafter, a synthetic medium was formulated on the basis of a chemical analysis of insect hemolymph (11, 12); however, this still needed to be supplemented with insect hemolymph (13). Currently, insect cell culture media are supplemented with fetal bovine serum (FBS) instead of insect hemolymph as FBS has been proven to be beneficial for the growth of insect cells. In a previous study, it was shown that the production of recombinant protein in an insect cell-baculovirus system is improved by supplementing the medium with silkworm hemolymph (14). This article reports that silkworm hemolymph inhibits baculovirus-induced insect cell apoptosis.

MATERIALS AND METHODS

Cell culture. *Spodoptera frugiperda* (Sf9) cells were cultivated in a Grace medium (Gibco) supplemented with 5% fetal bovine serum (FBS, Gibco), 0.35 g/liter $NaHCO_3$, and antibiotic–antimicotic (Gibco). The cells were maintained at 28°C. Silkworm hemolymph was added to the medium to investigate its effect on the host cell apoptosis.

Collection of silkworm hemolymph. The silkworm hemolymph was collected from the fifth instar larvae by clipping the side of an abdominal leg. The collected hemolymph was heat-treated at 60°C for 30 min, then chilled and centrifuged (15). The supernatant was filtered with a 0.2 μ membrane filter and used for supplementing the medium.

Infection with baculovirus. Cells in the late exponential growth phase (5 days in culture) were infected with a recombinant baculovirus. The recombinant baculovirus used was the *Autographa californica* nuclear polyhedrous virus (AcNPV) that produces β-galactosidase under the control of a polyhedrin promoter. For the infection, the medium was aspirated and a virus stock solution added. A multiplicity of infection (MOI) of 13 was used for all experiments. After incubating for 1 h, the virus solution was replaced with the medium used before the infection.

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Measurement of cell viability. The cell concentration was measured using a hemocytometer and viable cells were detected by the trypan blue exclusion test. Since dead cells absorb trypan blue (Sigma), they can be identified under light microscopy. The cell viability was defined by the ratio of viable cells to total cells.

Measurement of cloned gene expression. The activity of β-galactosidase was assayed by measuring the hydrolysis of *o*-nitrophenyl-β-D-galactoside (ONPG) (16). The extracellular activity was determined from the supernatant of the sample after centrifugation. To measure the intracellular activity, the precipitated cells were washed twice with PBS (phosphate-buffered saline), then the resuspended cells in the PBS were disrupted at 0°C using a ultrasonic homogenizer (US-150T, Nissei). The cell debris was removed by centrifugation at 4200*g* for 5 min. The intracellular activity of β-galactosidase was thus determined from the supernatant of the cell extract. The expression of β-galactosidase was then calculated by dividing the total activity, that is the sum of the intracellular and extracellular activities, by the total cell number.

Analysis of cellular and viral proteins. Cells were washed 3 times with phosphate-buffered saline (pH 6.2) and lysed with sodium dodecyl sulfate (SDS) gel loading buffer (2% SDS). Cell lysate obtained from 3×10^5 cells and 7.5% polyacrylamide gel were used for SDS-polyacrylamide gel electrophoresis.

Apoptosis assay. For the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method, TUNEL Label (Cat. No. 1767291) and TUNEL Enzyme (Cat. No. 1767305) were purchased from Boehringer Mannheim, Germany, to prepare the TUNEL reaction mixture. Cell fixation, permeabilization, and labeling were all carried out according to the manufacturer's standard procedures. The cells labeled by the TUNEL assay were analyzed by fluorescence microscopy (TE300, Nikon) with a B-filter. For an assay of the DNA fragmentation, cellular DNA was extracted using a previously reported method (17). The DNA fragmentation pattern was then examined by electrophoresis in a 2% agarose gel.

RESULTS AND DISCUSSION

After the baculovirus infection, the total cell density remained at a constant level, whereas the viable cell density began to decrease exponentially 3 days after the infection. The host cell viability defined by the ratio of viable cells to total cells is shown in Fig. 1. The viability remained at a high level for 3 days after infection in the media supplemented with 5% or 10% FBS. With the addition of silkworm hemolymph, the longevity of the host cells increased (18); i.e., the host cell viability remained at a high level for 6 days after infection in the medium supplemented with 5% FBS and 5% silkworm hemolymph.

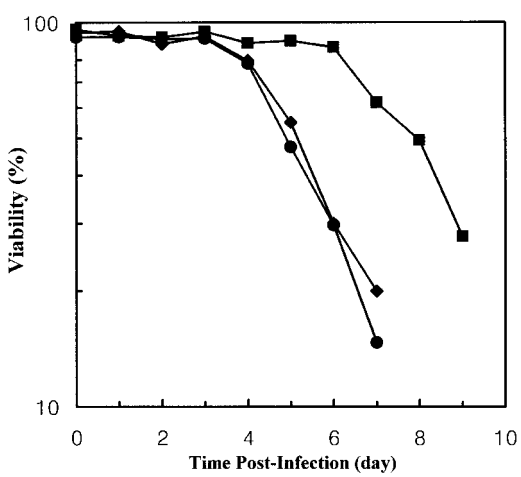

FIG. 1. Effect of silkworm hemolymph on host cell longevity. The host cell viability was defined by the ratio of viable cells to total cells. ◆, 5% FBS; ●, 10% FBS; ■, 5% FBS and 5% silkworm hemolymph.

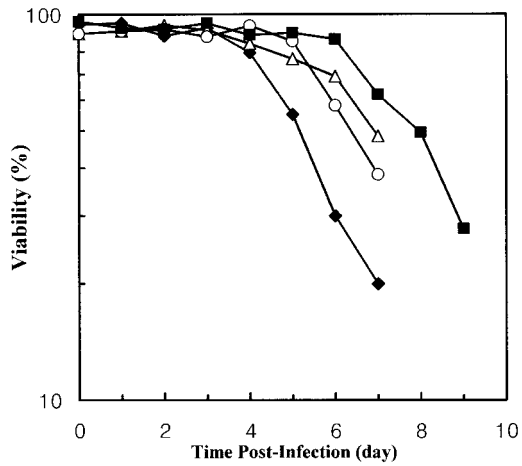

FIG. 2. Addition of silkworm hemolymph after infection was less effective on the delay of cell death. Every medium was supplemented with 5% FBS. ◆, without silkworm hemolymph; ■, medium was also supplemented with 5% silkworm hemolymph before inoculation; △, 5% silkworm hemolymph was added after infection; ○, 5% silkworm hemolymph was added every day after infection. The medium volume was maintained at a constant level by removing a corresponding volume of the medium before each addition.

When the cells were cultured in a medium supplemented with silkworm hemolymph before the inoculation, the host cell longevity increased as shown in Fig. 1. However, if the silkworm hemolymph was added after the infection, it had less effect on the host cell longevity as shown by the open symbols in Fig. 2. In the former case, the exposure to the silkworm hemolymph was long enough for the cells to absorb the apoptosis-inhibiting components before the infection. In the latter case, the silkworm hemolymph was added 1 h after incubation with the virus solution. Accordingly, the cells only began to absorb the silkworm hemolymph components after the infection. Apoptosis is inhibited as the virus enters the late phase of infection (2, 19, 20). This is a pivotal time during a virus infection because DNA replication initiates and appears to activate late gene expression and the shut-off of host gene expression (21).

The host cell longevity increased further when the cells were cultured in a medium supplemented with 10% silkworm hemolymph before the inoculation, as shown in Fig. 3. To observe the effect of the addition time, 5% more silkworm hemolymph was added at various times after the cells were cultured in media containing 5% FBS and 5% silkworm hemolymph. Fig-

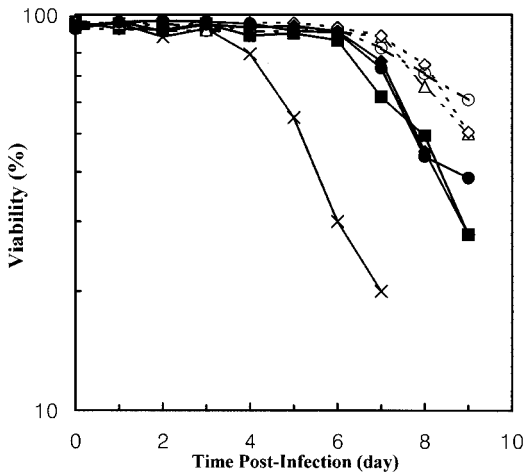

FIG. 3. Addition of silkworm hemolymph during infection was the most effective in delaying cell death. Every medium was supplemented with 5% FBS. ×, without silkworm hemolymph; ■, medium was also supplemented with 5% silkworm hemolymph before inoculation; △, 5% silkworm hemolymph was added after infection. ◇, ○, ●, ◆: Basal medium contained 5% FBS and 5% silkworm hemolymph. ◇, 5% more silkworm hemolymph was added one day before infection. ○, 5% more silkworm hemolymph was added during infection. ●, 5% more silkworm hemolymph was added one day after infection. ◆, 5% more silkworm hemolymph was added 3 days after infection.

ure 3 shows that addition either before or during the infection was effective (open symbols); however, addition after the infection was not so effective. This difference may be explained by a higher transfer rate of silkworm hemolymph into the cells during the virus internalization step. There are two distinct routes by which a virus may enter a cell: endocytosis or fusion. A baculovirus enters a cell largely by endocytosis, although fusion at the plasma membrane cannot be ruled out (22). This endocytosis enhances the transfer of silkworm hemolymph into a cell, consequently, the addition of silkworm hemolymph either before or during the infection will be more effective than after the infection.

A baculovirus first attaches to the host cell surface and then enters the cell. Most viruses attach to cells through a highly specific binding between surface proteins on the virus and cell surface receptor molecules on the host cell membrane (22), although the insect cell receptor that mediates a baculovirus attachment and infection has not yet been identified. Nonetheless, the delayed cell death due to silkworm hemolymph is not caused by the inhibition of these attachment and internalization steps, as silkworm hemolymph was still effective even when it was added after the infection step, as shown in Fig. 2. It was also found that the number of hypothetical targets, considered as a measure of the host cell susceptibility to a baculovirus (23), was not affected by silkworm hemolymph (data not shown). The n-target inactivation model that was mathematically derived to explain the survival rate of cells upon irradiation (24) was then applied to the baculovirus-induced insect cell death (23).

The virus infection procedure used in the experiments also confirms that the delayed cell death due to silkworm hemolymph was not due to an inhibition of the virus attachment and internalization steps. For the infection, the medium was aspirated and the cells were incubated with a baculovirus solution which does not contain silkworm hemolymph. Then the virus solution was then aspirated and replaced with the medium containing silkworm hemolymph. According to this procedure, there was no silkworm hemolymph in the solution during the infection step, except the case of the addition of silkworm hemolymph during the infection. This confirms that the delayed cell death is not due to the inhibition of infection by silkworm hemolymph. The increase in the cloned gene expression with the addition of silkworm hemolymph is further evidence that silkworm hemolymph does not inhibit the infection (see Fig. 4). If silkworm hemolymph inhibits the infection, the expression of the β-galactosidase gene, which was cloned into the baculovirus DNA, should have decreased. Figure 4 shows that silkworm hemolymph not only increased the expression rate of the cloned gene but also increased the period of production.

Cells undergoing apoptosis activate an endonuclease that cleaves the DNA between the nucleosomes to produce fragments. This DNA digestion is the biochemical hallmark of apoptosis. DNA fragmentation was observed from the fourth day after infection when the cells were incubated in a medium without silkworm hemolymph. In contrast, no DNA fragments were detected until the sixth day when the culture medium

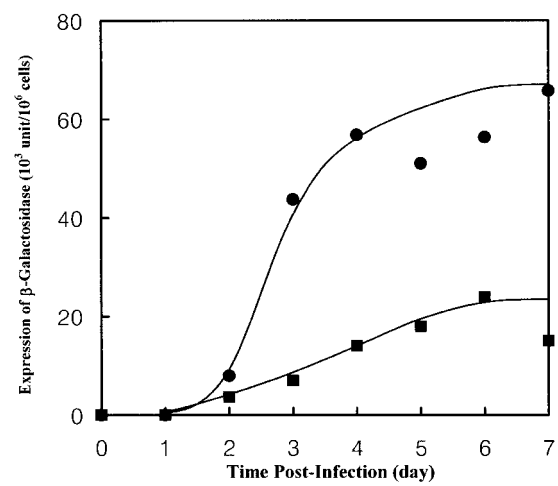

FIG. 4. Silkworm hemolymph increased the cloned gene expression. The β-galactosidase expression was determined by dividing the total activity, that is the sum of the intracellular and extracellular activities, by the total cell number. Every medium was supplemented with 5% FBS. ■, without silkworm hemolymph; ●, medium was also supplemented with 5% silkworm hemolymph before inoculation.

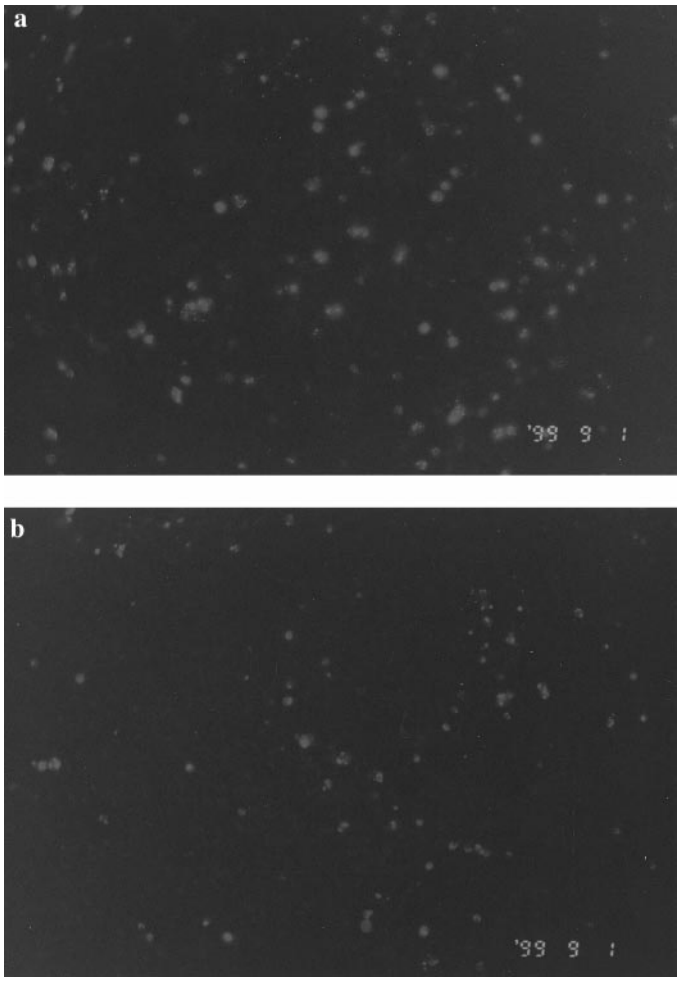

FIG. 5. Inhibition effect of silkworm hemolymph on DNA strand breaks. Cells were either not treated (a) or treated (b) with 5% silkworm hemolymph in the infection step. The cells were labeled on the 4th day after infection with fluorescein-dUTP by using exogenous terminal deoxynucleotidyltransferase (TdT) (magnification, ×200).

was supplemented with silkworm hemolymph (data not shown). In each case, the time when DNA digestion began corresponded to the time when the viability began to decrease (Fig. 1).

The inhibition of apoptosis by silkworm hemolymph was confirmed by the TUNEL assay. In this assay, DNA strand breaks are identified by fluorescence. The baculovirus-infected cells cultured in a medium without silkworm hemolymph (Fig. 5a) presented more fluorescence than those treated with silkworm hemolymph during the infection (Fig. 5b). The results show that baculovirus-induced insect cell apoptosis was significantly delayed by silkworm hemolymph. One possible explanation for this phenomenon is that silkworm hemolymph contains apoptosis-inhibiting components. Another possibility is that silkworm hemolymph increases the expression of the baculovirus gene *p35* which is known to prevent an apoptotic response. Figure 6 shows the results of SDS-polyacrylamide gel electrophoresis. The increase of the cloned β-galactosidase gene expression in the medium supplemented with silkworm hemolymph (lanes 5 and 6, see arrows) supports this possibility. Figure 6 also shows that the production of other viral proteins, which were not detected before infection (lane 1), were enhanced when silkworm hemolymph was added to the culture medium (lanes 5 and 6, see arrows). However, the silkworm hemolymph did not cause any difference in the expression of cellular proteins before infection (data not shown). Further study is required to elucidate the mechanism for the inhibition of apoptosis by silkworm hemolymph.

To investigate the heat stability of the substance in silkworm hemolymph, the hemolymph was heat-treated at various temperatures for 30 min. After chilling and centrifuging it, the supernatant was used as the medium supplement. Cell did not grow in the medium supplemented with intact hemolymph without heat treatment (25). The intact silkworm hemolymph and even the hemolymph heat-treated at 50°C darkened visibly during the incubation due to the activity of tyrosinase in hemolymph, producing melanin via intermediary quinones (26). The production of toxic quinones in the medium consequently inhibits the cell growth. Therefore, the hemolymph heat-treated at 60°C was used as a control. The hemolymph heat-treated at the higher temperatures was less effective than that at 60°C as shown in Fig. 7. The silkworm hemolymph heat-treated at 70°C for 5, 10, or 20 min was also less effective than that at 60°C for 30 min (data not shown). The purification and identification of the effective substance in silkworm hemolymph are in progress. According to our preliminary results, the molecular weight of the substance is higher than 20 kDa.

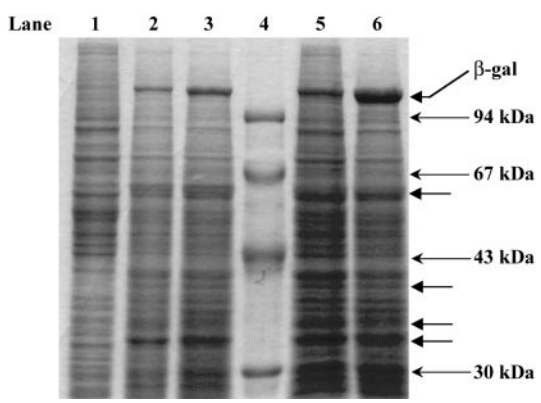

FIG. 6. Silkworm hemolymph enhanced the production of viral proteins. Cell lysate obtained from 3×10^5 cells and 7.5% polyacrylamide gel were used for SDS–polyacrylamide gel electrophoresis. Lane 1, before infection; lane 2, 2 days after infection in 10% FBS medium; lane 3, 3 days after infection in 10% FBS medium; lane 4, marker (Size is indicated in the margin); lane 5, 2 days after infection in 5% FBS/5% hemolymph medium; lane 6, 3 days after infection in 5% FBS/5% hemolymph medium.

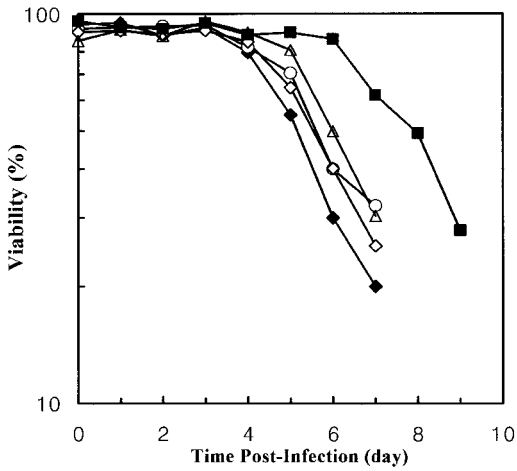

FIG. 7. Heat stability of the substance in silkworm hemolymph. Medium was supplemented with 5% FBS and 5% silkworm hemolymph heat-treated at various temperatures for 30 min. ◆, without silkworm hemolymph; ■, with silkworm hemolymph heat-treated at 60°C; △, with silkworm hemolymph heat-treated at 70°C; ○, with silkworm hemolymph heat-treated at 80°C; ◇, with silkworm hemolymph heat-treated at 90°C.

Silkworm hemolymph can be used for the efficient production of recombinant proteins in the insect cell-baculovirus system, since host cell viability is important for the replication of the baculovirus DNA containing a recombinant gene and the expression of the cloned gene. Recently, we have also observed similar results in a mammalian cell-animal virus system. Accordingly, the inhibition of apoptosis using silkworm hemolymph may lead to new approach to the minimization of cell death during commercial animal cell culture.

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