Porphyromonas gingivalis Peptidoglycans Induce Excessive Activation of the Innate Immune System in Silkworm Larvae*

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Porphyromonas gingivalis, a pathogen that causes inflammation in human periodontal tissue, killed silkworm (Bombyx mori, Lepidoptera) larvae when injected into the blood (hemolymph). Silkworm lethality was not rescued by antibiotic treatment, and heat-killed bacteria were also lethal. Heat-killed bacteria of mutant strains lacking virulence factors also killed silkworms. Silkworms died after injection of peptidoglycans purified from P. gingivalis (pPG), and pPG toxicity was blocked by treatment with mutanolysin, a peptidoglycan-degrading enzyme. pPG induced silkworm hemolymph melanization at the same dose as that required to kill the animal. pPG injection increased caspase activity in silkworm tissues. pPG-induced silkworm death was delayed by injecting melanization-inhibiting reagents (a serine protease inhibitor and 1-phenyl-2-thiourea), antioxidants (N-acetyl-l-cysteine, glutathione, and catalase), and a caspase inhibitor (Ac-DEVD-CHO). Thus, pPG induces excessive activation of the innate immune response, which leads to the generation of reactive oxygen species and apoptotic cell death in the host tissue.

The immune system is crucial for animal self-defense against pathogenic microorganisms. On the other hand, excessive activation of immune responses may cause serious damage to the host. Severely infected human patients die of septic shock and multiple organ failure, which seem to be caused by excessive activation of the host immune systems by the pathogens. The underlying pathologic mechanisms of sepsis, however, are not clear, and effective antiseptic remedies are yet to be established.

In vertebrates, the immune system is divided into two categories: innate immunity and acquired immunity. Because innate immunity is the front line of host defense against pathogens and is involved in initiating acquired immune responses, it is a prominent target in studies of the pathology of diseases caused by immune system deregulation. Most of the major components of the innate immune system are conserved from vertebrates to invertebrates (1). Thus, invertebrate models with simple biologic systems are useful for studying the activation mechanisms of innate immune responses, but there are no reports of pathologic invertebrate models that are killed by excessive innate immune responses caused by bacterial invasion of the bloodstream.

The silkworm, Bombyx mori, is an invertebrate model that can be administered quantitative injections of pathogens and drugs (2–4) and is suitable for the study of innate immunity (5). We recently reported that peptidoglycans and glucans, the cell wall components of bacteria and fungi, respectively, trigger the generation of reactive oxygen species (ROS)2 from silkworm blood cells (hemocytes) followed by the activation of serine proteases and induce the activation of paralytic peptide (PP), an insect cytokine (6). At the same time, peptidoglycans and glucans induce melanization, a polymerization reaction of melanin that involves serine proteases (7, 8), and phenoloxidase (a copper-containing monoxygenase tyrosinase) in the insect plasma (9). Polymerized melanin physically surrounds the pathogens and blocks their growth. Moreover, ROS are generated as byproducts in the course of the melanization process (10–12) and are responsible for killing the pathogens. Although ROS production is important for self-defense, overactivation of ROS can be toxic to host animals. In the present report, we demonstrate that excessive activation of innate immune responses induced by Porphyromonas gingivalis, a human periodontal pathogen, is lethal in silkworms. To our knowledge, this is the first report that systemic bacterial challenge induces overactivation of the innate immune system, leading to host killing in invertebrates. This pathologic model could be applied to study the central mechanisms of inflammation in vertebrates and to evaluate therapeutic agents against immune dysfunction.

EXPERIMENTAL PROCEDURES

Reagents—p-(Aminophenyl)methanesulfonyl fluoride (APMSF) and 1-phenyl-2-thiourea (PTU) were purchased from Wako Pure Chemical Industries, Ltd. l-3,4-Dihydroxyphenylalanine (l-DOPA), mutanolysin, N-acetyl-l-cysteine, glutathione, catalase, N-acetyl-Asp–Glu–Val–Asp–al (Ac-DEVD-CHO), and L-3,4-Dihydroxyphenylalanine were purchased from Genome Pharmaceuticals Co. Ltd.

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The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables S1–S3 and Figs. S1–S10.

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The abbreviations used are: ROS, reactive oxygen species; PP, paralytic peptide; APMSF, p-(aminophenyl)methanesulfonyl fluoride; PTU, 1-phenyl-2-thiourea; l-DOPA, l-3,4-dihydroxyphenylalanine; pPG, peptidoglycan purified from P. gingivalis; NAC, N-acetyl-l-cysteine; DAP, diaminopimelic acid.

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melanin, and \textit{Staphylococcus aureus} peptidoglycan were purchased from Sigma-Aldrich. Hydrogen peroxide was purchased from Kanto Chemical Co., Inc. \textit{P. gingivalis} LPS was purchased from Invitrogen. \textit{Escherichia coli} peptidoglycan was purchased from InvivoGen. The active form of PP, which was chemically synthesized (13), and anti-PP antisera (14) were kindly provided by Dr. M. Kamimura (National Institute of Agrobiological Sciences).

\textit{Bacteria—}\textit{P. gingivalis} ATCC33277 was anaerobically grown in WCA medium (1% tryptone, 1% gelatin peptone, 0.5% yeast extract, 0.1% glucose, 0.5% NaCl, 0.1% arginine, 0.1% pyruvic acid, 0.5 \(\mu\)g/ml menadione, 5 \(\mu\)g/ml hemin, pH 7.2) at 37°C. \textit{P. gingivalis} mutants KDP129, KDP133, KDP136, and KDP137 lacking proteases were previously described (15). Construction of the \textit{P. gingivalis mjfa1 fimA} mutant (KDP155) lacking fimbiae is described in the \textit{supplemental information}.

\textit{Closstridium perfringens} ATCC13124 and \textit{Propionibacterium acnes} ATCC6919 were obtained from the RIKEN Bioresource Center. \textit{C. perfringens} was anaerobically grown in a broth (0.4% yeast extract, 1.5% proteose peptone, 0.4% sucrose for density gradient centrifugation (Nacalai Tesque) followed by incubation at room temperature. The sample was centrifuged (43,000 \(\times\) g, 1 h, 12°C) and washed twice in water. The precipitate was suspended in saline and sonicated using a Sonifier 450 (Branson) with the output control of 1 for 5 s.

\textit{Muscle Contraction Assay—}The measurement of muscle contraction activity in silkworms was performed as described previously (6, 19).

\textit{In Vitro Detection of the Active Form of PP in Isolated Hemolymph—}Hemolymph collected from cutting the abdominal legs of fifth instar larvae was incubated with test samples at 25°C for 3 min and then boiled for 5 min. The samples were centrifuged at 10,000 \(\times\) g for 10 min and subjected to Western blot analysis. Conditions of Western blot analysis to detect active PP in isolated hemolymph were the same as those described previously (6).

\textit{Melanization Assay—An \textit{in vitro} PO assay was performed as previously described (20) with slight modification. Fifth instar larvae (day 4) were injected with 100 \(\mu\)l of a 200 mM sucrose solution (sucrose for density gradient centrifugation (Nacalai Tesque) dissolved in physiologic saline containing 110 mM KCl, 4 mM NaCl, 15 mM MgCl\(_2\), 4 mM CaCl\(_2\), and 5 mM potassium phosphate) followed by incubation at room temperature for 1 h, and the hemolymph was collected in ice-cold tubes. Hemo-lymph obtained from each larva was centrifuged at 300 \(\times\) g for 5 min, and the aliquot that remained a transparent yellowish color was further used as S-plasma. Ten microliters of each test sample (50 \(\mu\)g/ml of pPG with or without inhibitors) was mixed with 20 \(\mu\)l of S-plasma and 70 \(\mu\)l of a reaction solution (4 mM L-DOPA in 80 mM potassium phosphate buffer, pH 6.0) and incubated at 30°C for 1 h. The \(A_{490}\) values were measured to assess the amount of melanin. For the \textit{in vivo} melanization assay, 50 \(\mu\)l of each sample was injected into the hemolymph of silkworm larvae, and the larvae were incubated at 27°C for 1 h. Each hemolymph sample was diluted in insect physiologic saline (150 mM NaCl, 5 mM KCl, and 1 mM CaCl\(_2\)) containing 1 mM PTU to prevent further melanization. The \(A_{490}\) values were measured to assess the amount of melanin.
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Mutanolysin Treatment—pPG was incubated with 100 μg/ml mutanolysin (930 units/ml) in PBS at 37 °C for 2–3 h and then boiled for 5 min. For mock treated groups, pPG was incubated without mutanolysin in PBS and then boiled as above.

Therapeutic Effects of Reagents against pPG-induced Silkworm Death—Reagents dissolved in 0.9% NaCl (saline) or PBS were filtered through a sterile 0.22-μm polyvinylidene difluoride filter (Millipore) and mixed with pPG. One of each test sample (50 μl) was injected into the hemolymph of silkworm larvae (day 2 of fifth instar) with a 1-ml syringe attached to a 27-gauge needle (Terumo). Injected larvae were incubated at 27 °C, and the number of viable larvae was counted. For statistical analysis, the data were plotted using the Kaplan-Meier method with Prism 5 (GraphPad Software, Inc.) and tested for significance using the log rank test. In experiments that were repeated using independent preparations of purified pPG, the median survival times of larvae in independent assays are summarized in the supplemental tables.

Measurement of Lactate Dehydrogenase Activity—Fifty microliters of PBS or pPG (50 ng/larva) were injected into silkworms (day 2 of fifth instar), and the hemolymph was collected 24 h later. Hemolymph was centrifuged to obtain plasma fractions. Five microliters of plasma were mixed with 1 ml of reaction solution (0.6 μM l-alanine and 0.2 mM NADH in 0.1 M Tris-HCl, pH 7.5) and 100 μl of 0.1 M Tris-HCl or pyruvate solution (33 mM pyruvate in 0.1 M Tris-HCl, pH 7.5) and incubated at 30 °C for 60 min. Decreases in the A405 values of samples incubated with pyruvate were determined to assess the lactate dehydrogenase activity.

Caspase Activity Assay—Detection of caspase activity was performed by APOPTO Caspase-3 colorimetric assay kit (code no. 4800; MBL). Three to four larvae (day 2 of fifth instar) were injected with saline or pPG (50 ng/larva) and incubated at 27 °C for 8 h. The heads of the larvae were cut off, and the peritrophic membranes were removed with the intestinal contents. Whole tissue from each larva was homogenized in an ice-cold tube containing 1 ml of lysin buffer. The homogenates were centrifuged at 15,300 × g for 5 min at 4 °C, and the supernatant was further centrifuged at 10,000 × g for 5 min at 4 °C. Protein concentrations of the aliquots were quantified with a Coomassie Plus™ protein assay reagent (Thermo Scientific) using bovine serum albumin as the standard, and samples were adjusted to 4 mg of protein/ml. Twenty-five microliters of each sample were mixed with an equal volume of reaction buffer containing 100 mM dithiothreitol, and 2.5 μl of 10 mM DEVD-p-nitroanilide (a caspase substrate) was added. The samples were incubated at 37 °C for 12 h, and the A405 values were measured to assess the amount of free p-nitroanilide. To assess the caspase activity, caspase-dependent increases in A405 were calculated by subtracting the A405 value between each sample incubated in the presence and absence of 10 μM DEVD-fmk, a pharmacologic caspase inhibitor.

Measurement of IL-6 Produced by Murine Peritoneal Macrophages—Murine peritoneal macrophage production of IL-6 was assessed as previously reported (21). Briefly, mice (C57BL/6) were intraperitoneally injected with Brewer thiglycollate medium (Kanto Chemical Co., Inc.), and peritoneal macrophages were collected 3 days later. Macrophages were incubated with samples in RPMI 1640 medium (Invitrogen) at 37 °C for 3 days. Quantification of IL-6 in the culture medium was performed using the Ready-SET-Go! mouse IL-6 ELISA kit (eBioscience).

RESULTS

Silkworm Killing Effect of P. gingivalis—Silkworm larvae are susceptible to various bacteria that are pathogenic to mammals (3); thus, the silkworm infection model is useful for screening antibiotics based on their therapeutic effects in vivo (22). Injection of P. gingivalis killed silkworms, but antibiotics were not effective against P. gingivalis infection, despite their effectiveness against other pathogenic bacteria such as S. aureus (22). Injection of a suspension of anaerobically cultured P. gingivalis (ATCC33277) into the hemolymph of silkworm larvae killed 50% of the larvae within 90 h (Fig. 1A). The color of the sil-
worm hemolymph infected with *P. gingivalis* turned dark brown before death. As we previously reported (22), death of *S. aureus*-infected silkworms is prevented by treatment with tetracycline (Fig. 1B and supplemental Table S1). In contrast, tetracycline had no therapeutic effect on silkworms infected with *P. gingivalis* (Fig. 1A and supplemental Table S1). The minimum inhibitory concentration of tetracycline against *P. gingivalis* was 0.2 μg/ml, which was lower than that against *S. aureus* (1 μg/ml). Although the number of bacteria in the hemolymph decreased during the time course of infection (supplemental Fig. S1), silkworms showed high mortality when infected with *P. gingivalis*. Moreover, autoclaved cells of *P. gingivalis* and live *P. gingivalis* bacteria killed silkworms over a similar time course (Fig. 1C and supplemental Table S2), indicating that *in vivo* growth of *P. gingivalis* is not required to kill silkworm larvae.

Silkworm Killing Effect of pPG—Next, we attempted to identify the components of *P. gingivalis* responsible for killing silkworms. Proteases and fimbriae produced by *P. gingivalis* cause inflammation in mammalian models (15, 23–25). We tested the killing effects of heat-treated mutant strains of *P. gingivalis* that lack genes encoding these virulence factors. Mutants and wild-type *P. gingivalis* killed silkworms with a similar time course (supplemental Fig. S2). Because autoclaved *P. gingivalis* cells retained its killing activity against silkworms, we hypothesized that surface components of *P. gingivalis* induce the excess activation of immune responses and the subsequent death of silkworms. In general, bacterial components such as peptidoglycans and LPSs are potent stimulants of innate immunity. Injection of pPG into silkworm larvae killed the larvae in a dose-dependent manner (Fig. 2A). In addition, pPG was toxic at other developmental stages of *B. mori*, such as pupal (supplemental Fig. S3) and adult moth stages (data not shown). Furthermore, the killing effect of pPG in silkworm larvae was abolished by treatment with mutanolysin, an enzyme that degrades peptidoglycans (Fig. 2B). Although an LPS fraction obtained from *P. gingivalis* killed the larvae, its toxicity was attenuated by mutanolysin treatment (supplemental Fig. S4), suggesting that the killing effect of the LPS fraction was due to contaminated peptidoglycans. Therefore, we focused on peptidoglycans as the factor responsible for *P. gingivalis* pathogenesis.

Minor Involvement of the Activation of Insect Cytokine PP in pPG-induced Killing of Silkworms—We recently reported that injection of *S. aureus* peptidoglycans triggered the activation of PP, an insect cytokine involved in self-defense, in silkworm hemolymph (6). Because active PP induces muscle contraction in silkworms, the immunostimulatory activities of exogenous substances can be quantified by measuring the length of larval specimens (6). Injection of autoclaved cells of *P. gingivalis* into larval specimens dose-dependently induced muscle contraction (supplemental Fig. S5A). Because ~10 min was required to induce the maximum contraction of larval specimens, the reaction was not likely due to excitation of the nervous system, which occurs within 2 s (19). Incubation of pPG in isolated hemolymph led to the *in vitro* generation of active PP (supplemental Fig. S5B). Therefore, pPG also induces PP activation in the silkworm hemolymph.

An overdose of active PP kills silkworms (26); therefore, we hypothesized that death of silkworms after pPG injection was due to excess activation of PP. Injection of anti-PP antiserum, which inhibits the activation process of PP (6, 13), did not attenuate the lethal effect of pPG (supplemental Fig. S5C). Therefore, we concluded that silkworm death after pPG injection was caused by a mechanism other than PP overactivation.

Involvement of Hemolymph Melanization in the pPG- and *P. gingivalis* Live Bacteria-induced Killing Effect—Bacterial peptidoglycan is a well known inducer of hemolymph melanization in insects (9). Melanization involves cascade reactions of serine proteases followed by the activation of PO. pPG induced pigment formation in an *in vitro* PO assay containing silkworm plasma and L-DOPA as a substrate (Fig. 3A), pPG-induced *in vitro* PO activation in the hemolymph sample was blocked by APMSF (a serine protease inhibitor) (Fig. 3A). Moreover, the *in vitro* PO activity was inhibited by PTU (Fig. 3A), consistent with a previous report (27). We further tested the stimulatory effect of pPG on melanization in vivo. A rapid blackening of the silkworm hemolymph was observed within 1 h of the injection of a lethal dose of pPG (Fig. 3B). Co-injection of APMSF or PTU suppressed the pPG-dependent melanization of the silkworm hemolymph (Fig. 3C), suggesting that *in vivo* melanization by pPG requires serine proteases and PO, as indicated by the *in vitro* assay (Fig. 3A). pPG treated with mutanolysin had a reduced ability to induce melanization of silkworm hemolymph *in vivo* (Fig. 3D). Furthermore, the minimum dose of pPG...
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required to induce in vivo melanization (5 ng/larva) corresponded to the minimum dose required to kill silkworms (Figs. 2A and 3E).

We next tested the effects of melanization inhibitors on pPG-induced silkworm lethality. Injection of APMSF or PTU with pPG reduced the silkworm mortality (Fig. 4, A and B), suggesting that melanization following the activation of serine proteases and PO is involved in pPG-induced silkworm death. A high dose of melanin (0.5 mg/larva), however, did not kill silkworms (data not shown); the amount of melanin was estimated to be 10-fold higher than that produced in the silkworm hemolymph after injection of pPG. Therefore, the lethal effect of pPG on silkworms is not likely to be due to the direct toxicity of melanin. Although there are no reports that hemolymph melanization induced by systemic infection causes death in invertebrates, highly reactive ROS are generated as byproducts in the melanization process (10–12). Thus, we assumed that ROS induce damage to both the host animal and the invading pathogens. Injection of hydrogen peroxide, a source of ROS, was toxic to silkworms (supplemental Fig. S6). On the other hand, co-injection of N-acetyl-L-cysteine (NAC), a radical scavenger, or antioxidants such as glutathione and catalase delayed pPG-induced silkworm death (Fig. 4, C and D). These data suggest that ROS produced in the course of melanization following pPG injection had lethal effects on silkworms.

Hemolymph melanization was observed after injection of a P. gingivalis (live or heat-killed bacteria) suspension as well as pPG. We further tested whether the lethal mechanism of pPG was applicable to the silkworm death caused by injection of P. gingivalis live bacteria. First, we examined the effect of APMSF, PTU, or mutanolysin on the induction of melanization in silkworm hemolymph caused by the injection of P. gingivalis live cells. The in vivo melanization induced by P. gingivalis bacterial culture was effectively blocked by both APMSF and PTU (supplemental Fig. S7A). Moreover, mutanolysin treatment of
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*P. gingivalis* live cells abolished their *in vivo* melanization stimulating activity (supplemental Fig. S7B). We next examined the effect of APMSF and NAC on the silkworm killing effect of *P. gingivalis* bacterial culture. Both reagents delayed the death of silkworms infected by *P. gingivalis* (supplemental Fig. S7, C and D). These results suggest that, as in the case of peptidoglycan fraction, live *P. gingivalis* cells are capable of killing silkworms via the activation of melanization, which leads to production of reactive oxygen species.

**Induction of Apoptosis in Silkworm Tissues by pPG—ROS** are known to induce cell death. Therefore, we tested whether pPG damages the cells in silkworm tissues. The pPG injection-induced release of lactate dehydrogenase in the plasma was under the limits of detection (<1.0 × 10^−2 units/μl), suggesting that the contribution of necrotic cell death is minor. We then examined the involvement of apoptotic cell death in pPG-induced silkworm killing. Signaling molecules in apoptotic pathways are conserved from vertebrates to invertebrates (28). Some caspases, a family of cysteine proteases that execute cell death, and IAP (inhibitor of apoptosis protein) have been identified in *P. gingivalis* bacterial culture extracts (Fig. 5A). Based on an *in vitro* caspase assay, injection of pPG induced an increase in caspase activity in whole larval extracts (Fig. 5A). Furthermore, a pan-caspase inhibitor (Ac-DEVD-CHO) delayed the deaths of pPG-injected silkworms (Fig. 5B). The killing effect of *P. gingivalis* bacterial culture was also inhibited by the co-injection of Ac-DEVD-CHO.

**FIGURE 4. Effects of reagents on silkworm death induced by pPG.** pPG (50 ng/larva) mixed with each reagent was injected into 10 larvae/group. *A*, effect of a serine protease inhibitor, APMSF (1 mM or 10 mM). Differences in the survival plots in pPG alone and pPG with 1 mM APMSF or in pPG alone and pPG with 10 mM APMSF were statistically significant (*p* < 0.001, or *p* < 0.0001, respectively). *B*, effect of a PO inhibitor, PTU (1 or 10 mM). Differences in the survival plots in pPG alone and pPG with 10 mM PTU were statistically significant (*p* < 0.001, or *p* < 0.0001, respectively). *C*, effect of a radical scavenger, NAC (1 or 10 mM). Differences in the survival plots in pPG alone and pPG with 10 mM NAC were statistically significant (*p* < 0.05 or *p* < 0.001, respectively). *D*, effect of glutathione (1 mg/ml) or catalase (2.5 mg/ml). Differences in the survival plots between pPG alone and pPG with glutathione or in pPG alone and pPG with catalase were statistically significant (*p* < 0.001, or *p* < 0.0001, respectively). The data shown are representative of three experiments using independent lots of purified pPG.

**FIGURE 5. Activation of caspase in larval tissues induced by pPG, and the effect of a caspase inhibitor on silkworm survival.** *A*, measurement of caspase activities in silkworm tissues. Saline or pPG (50 ng/larva) was injected into three or four larvae, and the larvae were incubated at 27 °C for 8 h. Whole tissue of each larva was homogenized and centrifuged. An aliquot of each sample, adjusted to have an equal protein concentration, was mixed with a substrate solution containing DEVD-p-nitroanilide and incubated at 37 °C for 12 h, followed by measurement of OD_{405} values (OD_{405}) to assess the amount of free p-nitroanilide. To assess the caspase activities, caspase-dependent increases in OD_{405} were calculated by subtracting the OD_{405} value between each sample incubated in the presence and absence of 10 μM DEVD-fmk, a pharmacologic caspase inhibitor. The data represent the means ± S.D. of three or four larvae, and statistical significances were determined by one-way analysis of variance test. *, *p* < 0.0001. *B*, 10 larvae were injected with pPG (50 ng/larva) or a mixture of pPG and the caspase inhibitor Ac-DEVD-CHO (10 μM or 100 μM). Differences in the survival plots between pPG alone and pPG with 10 μM DEVD or in pPG alone and pPG with 100 μM DEVD were statistically significant (*p* < 0.05 or *p* < 0.005, respectively). The data were plotted using the Kaplan-Meier method and tested for significance using the log rank test. The data shown are representative of three experiments using independent lots of purified pPG, and the median survival times of larvae in three independent assays are summarized in supplemental Table S3.
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(supplemental Fig. S7E), suggesting that activation of the apoptotic pathways has a significant role in both pPG-induced and *P. gingivalis* live bacteria-induced silkworm death.

**Effects of the Combination of Reagents on pPG-induced Silkworm Killing**—To elucidate the contribution of each biologic step (melanization, ROS production, and apoptosis) to the silkworm killing effects of pPG, we further examined the effect of treating silkworms with a combination of inhibitors. We used APMSF (a serine protease inhibitor), NAC (an antioxidant), and Ac-DEVD-CHO (a caspase inhibitor). As shown in supplemental Fig. S8, combinations of APMSF and NAC or of APMSF and Ac-DEVD-CHO provided no additional delay of silkworm killing by pPG compared with APMSF alone. Therefore, we concluded that ROS production and apoptosis are the major causes of silkworm death involving excessive melanization. Because we observed that combinations of the above reagents significantly delayed but did not completely prevent pPG-dependent larval death (supplemental Fig. S8), we could not rule out the possible involvement of other processes in the silkworm killing effect.

**Effects of Peptidoglycans from Other Bacteria on Hemolymph Melanization and Silkworm Killing**—In general, bacterial peptidoglycans are categorized by the chemical properties of the components. Most Gram-positive and Gram-negative bacteria possess Lys- and DAP-type peptidoglycans, respectively, based on the third amino acid of a muropeptide chain. Peptidoglycans of *P. gingivalis* contain L-L-DAP (31) instead of the L-D-(meso)-DAP contained by other Gram-negative bacteria such as *E. coli*. To evaluate the significance of the peptidoglycan type, we used L-Lys type peptidoglycans of *S. aureus*, L-D-(meso)-DAP type peptidoglycans of *E. coli*, or L-L-DAP type peptidoglycans purified from *C. perfringens* and *P. acnes* to test their abilities to stimulate silkworm killing and melanization. Peptidoglycans from *S. aureus* induced killing of silkworms and melanization at a relatively high dose (50 μg/larva) (Fig. 6). On the other hand, peptidoglycans from *E. coli* did not have lethal effects in silkworms, even at a high dose (50 μg/larva; data not shown). Moreover, the silkworm killing and hemolymph melanization effects of *C. perfringens* PG and *P. acnes* PG were weak compared with *P. gingivalis* PG at the same dose (Fig. 6). Therefore, we concluded that the L-L-DAP moiety was not sufficient for peptidoglycans to show high toxicity.

**pPG-induced Immune Activations in Other Animal Models**—To elucidate the generality of the phenomena observed above, we examined the lethal effects of pPG on other model animals. Injection of pPG into larvae of *S. littura* (tobacco cutworms), a Lepidopteran insect like *B. mori*, killed them in a dose-dependent manner (Fig. 7A). Moreover, pPG injection induced excessive hemolymph melanization and host killing in larvae of *Z. morio* (mealworms) (Fig. 7B) and *T. dichotomus* (Japanese horned beetles) (supplemental Fig. S9) that belong to the Coleoptera order. On the other hand, Dipteran insects such as *D. melanogaster* (Fig. 7, C and D) and *S. peregrina* (flesh flies) (Fig. 7E) were resistant to pPG-dependent killing, as the survival rates of both insects were not different between saline-injected and high dose pPG-injected groups. The amounts of pPG required to kill the insects were roughly calculated as follows: 2.5 ng/g of animal for *B. mori*, 100 ng/g of animal for *S. littura*, 5 μg/g of animal for *Z. morio*, 100 ng/g of animal for *T. dichotomus*, >65 μg/g of animal for *D. melanogaster*, and >50 μg/g of animal for *S. peregrina* (Figs. 2A and 7; supplemental Fig. S9). In addition, systemic blackening of the hemolymph was not observed in *D. melanogaster* and *S. peregrina* after pPG injection (data not shown), in contrast to the Lepidopteran and Coleopteran species described above. These results suggest that the concept of pPG-induced melanization and host killing could be expanded to other, if not all, insect genera.

**DISCUSSION**

*P. gingivalis* is a potent cause of periodontitis, and its presence is a risk factor for systemic inflammatory syndromes such as atherosclerosis and cardiac dysfunction (32). The ability of virulence factors such as proteases, fimbriae, and LPS to induce inflammation has been studied intensively (33, 34). In some cases, however, inflammation takes place independently of these factors, suggesting the existence of other immune stimulants (34). We found that pPG induced cell death in silkworm tissues via ROS production, which led to host death (Fig. 8). Moreover, pPG stimulated the production of IL-6 from mouse peritoneal macrophages (supplemental Fig. S10). These findings suggest that pPG, which acts as an immune stimulant in silkworms, also causes inflammation in mammals by overactivation of innate immune processes.
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The pPG-induced lethality of silkworm larvae was attenuated by treatments that inhibit hemolymph melanization; injection of a serine protease inhibitor or PTU, and incubation of pPG with mutanolysin. Therefore, we concluded that excess activation of melanization triggered by pPG resulted in silkworm death. In our experiments, melanization and caspase activation were observed within a few hours after injection, whereas larval death took a few days longer. This apparent time lag could be explained by the longer time required to disrupt physical homeostasis and induce dysfunction of most larval tissues than for excessive melanization and caspase activation.

Because APMSF, NAC, Ac-DEVD-CHO, or a combination of these reagents significantly delayed but did not completely prevent pPG-dependent death of the larvae (supplemental Fig. S8), we could not rule out the possible involvement of other cumulative effects of pPG injection in the larval killing mechanism.

In Drosophila, a mutant strain lacking serpin27A, which encodes a negative regulator of melanization, and a mutant that overproduces a peptidoglycan receptor, PGRP-LE, show frequent formation of melanotic pigments and high mortality rates during development (35–37); the causal relationship between melanization and fly death, however, is not clear. The serpin27A mutation does not affect susceptibility to systemic infection by the Gram-positive bacteria Micrococcus luteus or the Gram-negative bacterium E. coli (36, 37). Here, we showed that D. melanogaster larvae and adult flies were resistant to the lethal effects of pPG, in contrast to other insects such as S. litura (Lepidoptera), Z. morio (Coleoptera), and T. dichotomus (Coleoptera). Injection of pPG into B. mori and other insects susceptible to pPG-induced killing induced rapid and excessive melanization of the hemolymph throughout the whole body. On the other hand, D. melanogaster and S. peregrina larvae did not show such systemic melanization after pPG injection. Bidla et al. (38) reported that the wax moth Galleria mellonella (Lepidoptera) and D. melanogaster responded differently to injection of Bacillus megaterium peptidoglycans; widespread blackening of hemolymph occurred in G. mellonella, whereas melanization spots were located only at the injection site in D. melanogaster. The different responses to pPG among species could be explained by the evolutionary divergence of structures and functions of receptors or downstream factors including serpin27A, but further comparative studies are required to clarify this point. Recently, Ha et al. (39, 40) reported that oral Erwinia carotovora infection in Drosophila caused ROS production via the activation of dual oxidase in the midgut, resulting in fly mortality. To our knowledge, our results are the first to show that overactivation of systemic melanization induced by the invasion of pathogenic bacteria into the bloodstream leads to death in an invertebrate model.

Peptidoglycan, one of the main components of bacteria, is composed of cross-linked structures of peptide chains and polysaccharides containing N-acetylglucosamine and N-acetylmuramic acid. Length, composition, and modification of the peptide chains differ among bacterial species. In general, Gram-positive and Gram-negative bacteria possess L-Lys and L-D-
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(meso)-DAP, respectively, as the third amino acid of a muropeptide chain. On the other hand, muropeptides of P. gingivalis contain L,L-DAP instead of L,D- (meso)-DAP (31). In insects, the minimum peptidoglycan structure that stimulates antimicrobial peptide production has been identified (41–43). In contrast, the effect of differences in peptidoglycan structures on melanization is largely unknown. Here, we prepared peptidoglycans from C. perfringens and P. acnes that possess L,L-type peptidoglycans and tested their silkworm killing and melanization activities. Peptidoglycans from both bacterial species were less effective than P. gingivalis peptidoglycans for inducing silkworm killing and melanization. Therefore, we concluded that the high toxicity of P. gingivalis peptidoglycans could not be explained simply by the presence of the L,L-DAP moiety, and other structural characteristics specific to P. gingivalis (e.g. peptid chains with a high glycine content (44)) might be responsible. Further studies are required to determine the critical PG structure that causes excessive melanization and host killing.

Melanogenesis itself is a biologic reaction conserved among species in which the central enzyme tyrosinase converts tyrosine to DOPA. In humans, melanin synthesis occurs in skin pigment. Excess production of melanin induces dysplastic nevi, which is a known risk factor for malignant melanoma (45). Increased melanogenesis in dysplastic nevi leads to chronic generation of ROS as byproducts and oxidative DNA damage to nevus cells (46). Our finding that silkworms injected with pPG are killed by ROS in the course of melanization seems to indicate that common fundamental mechanisms underlie melanogenesis-related diseases in humans.

In severely infected mammals, ROS produced from immune cells cause tissue damage. Our results suggest that cell death induced by ROS in the course of the immune response causes death of the host animal in an invertebrate model, as in vertebrates. Furthermore, antioxidants such as N-acetyl-L-cysteine and glutathione, which have therapeutic effects in mammalian sepsis models (47), also delayed the pPG-induced death of silkworms. Therefore, we suggest that the silkworm-P. gingivalis infection model could be a useful in vivo screening system for compounds effective against sepsis caused by overactivation of the immune response.

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