Applying the silkworm model for the search of immunosuppressants

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SUMMARY Various stresses (high temperature, starvation, or sublethal Cryptococcal infection) increased the susceptibility of silkworms to bacterial infection by up to 100-fold, confirming the stress-induced immunosuppression reported in a range of species. When the silkworm was injected with a steroidal drug, betamethasone (1 mg/larva), the susceptibility of the silkworm to bacterial infection increased about 100-fold. This indicates that the immune function of the silkworm can be suppressed by a known compound that shows immunosuppressive effects in humans. We further tested the immunosuppressive effect of the culture supernatants (acetone extracts) of soil bacteria, and 24 out of 193 isolates showed the immunosuppressive activity. These results suggest that it is possible to search for immunosuppressive agents targeting innate immunity by using a silkworm bacterial infection model as a screening system, and that there may be candidate compounds for immunosuppressive agents among the substances produced by soil bacteria.

Keywords Silkworm model, immunosuppressants, screening system, natural products

1. Introduction

Immunosuppressive drugs are used in various inflammatory diseases and organ transplantation to suppress excessive immune responses (1). However, patients suffering from inflammatory diseases are still in need of new immunosuppressive compounds to improve their therapeutic efficacy. Currently used immunosuppressive agents can be classified into three categories: those that suppress the function of immunocompetent cells, those that suppress the production of cytokines, and those that exhibit cytotoxicity (1,2). In recent years, cyclosporine has been identified through in vitro screening of soil bacteria and is mainly administered during organ transplantation (3). In addition, antibody-based drugs targeting molecules expressed by immunocompetent cells are also being developed. However, since these screenings are mainly conducted in vitro, it is difficult to consider the pharmacokinetics and toxicity (ADMET) of the candidate compounds at the early stage of the screening, which poses a challenge for the research and development of immunosuppressive agents (1).

To solve the above problems, we have proposed a screening system for drug candidates by using silkworms (4-9). Screening of antimicrobial agents using a silkworm infection model has led to the identification of a new antimicrobial compound, Lysocin E, which has shown good therapeutic results in vertebrates (10,11). Furthermore, by using the silkworm model of hyperglycemia, we have discovered functional lactic acid bacteria that improves the blood glucose homeostasis (12-14). These findings indicate the usefulness of constructing a disease model in silkworm and conducting a large-scale exploration. In this study, we attempted to construct a model of immunosuppression in the silkworm with the aim of screening for new immunosuppressive agents.

2. Materials and Methods

2.1. Silkworms and injection technique

Silkworms were reared and injected with samples into the hemocoel by the method reported previously (15-17). Silkworm used in the experiment were 5th instar larvae, fed during day 1 (on the day of molt) and 2 (1 g/larva). Silkworms were utilized for experiments on day 2 (5th instar) unless otherwise specified.

2.2. Thermal stress and Staphylococcus aureus infection

Silkworms were injected with different doses of S. aureus MSSA1 strain and kept at 27°C (normal condition) or 37°C (high temperature condition). The silkworms were not fed during the infection experiment. The number
of surviving silkworms at 48 hours after infection was counted, and the LD\(_{50}\) value of MSSA1 strain for silkworms was estimated from the dose-action curve (see 'Estimation of LD\(_{50}\) values' section).

2.3. Starvation stress and \textit{S. aureus} infection

In the control group (no starvation stress), the silkworm was fed food (1 g/larva) on day 1 of the 5\(^{th}\) instar, and the infection experiment of \textit{S. aureus} (MSSA1 strain) was conducted using the silkworm on day 2. In the starvation-stressed groups, the silkworms were not fed after day 2 of the 5\(^{th}\) instar, and the same infection experiments as above were conducted either on day 3 (1 day of starvation), on day 4 (2 days of starvation), or on day 5 (3 days of starvation). The number of surviving silkworms at 48 hours after infection was counted, and the LD\(_{50}\) value of MSSA1 strain for silkworms was estimated from the dose-action curve (see 'Estimation of LD\(_{50}\) values' section) for each group.

2.4. Sublethal \textit{Cryptococcal} infection and \textit{S. aureus} infection

A group of silkworms (\textit{Cryptococcal} infection stress group) was injected with a 4-fold concentrated culture of \textit{C. neoformans} (50 µL/larva). The other group of silkworms (control group) was injected with the same volume of saline. Then silkworms of each group were injected with different doses of \textit{S. aureus} MSSA1 strain and kept at 27ºC. The number of surviving silkworms at 48 hours after infection was counted, and the LD\(_{50}\) value of MSSA1 strain for silkworms was estimated from the dose-action curve (see 'Estimation of LD\(_{50}\) values' section) for each group.

2.5. Betamethasone treatment and \textit{S. aureus} infection

Silkworms were injected with 10% DMSO (control group) or Betamethasone (betamethasone treated group) dissolved at a concentration of 20 mg/mL, and immediately afterwards injected with different doses of \textit{S. aureus} MSSA1 strain and kept without food at 27ºC. The number of surviving silkworms at 48 hours after infection was counted, and the LD\(_{50}\) value of MSSA1 strain for silkworms was estimated from the dose-action curve (see 'Estimation of LD\(_{50}\) values' section) for each group.

2.6. Estimation of LD\(_{50}\) values

The statistical programming language R (version 3.6.1) was used to estimate the LD\(_{50}\) values. The function 'drm' implemented in the package 'drc' ([https://cran.r-project.org/web/packages/drc/drc.pdf](https://cran.r-project.org/web/packages/drc/drc.pdf)) was used, and LL.3 was applied to the argument fct. From the results of the obtained model, LD\(_{50}\) estimates and their standard errors were read are described in the main text. The infected cell number was estimated from the injected volume: when overnight culture of MSSA1 contains 1 \times 10^{10} CFU/mL (i.e., 1 \times 10^{7} CFU/µL) of live cells, then the infected dose for injecting 50 µL of the culture will be \(5 \times 10^{5} CFU/larva\).

2.7. Brief search for immunosuppressive agents from a soil bacterial library

Fifty microliters of culture supernatant samples (acetone extracts) of 193 soil bacterial isolates were injected into the silkworm, and immediately afterwards, 50 µL of a 1000-fold diluted \textit{S. aureus} (MSSA1 strain) overnight culture was injected into the silkworm. There were three batches of bacterial isolates. Batch #1: 70 randomly isolated strains, Batch #2: 52 strains that formed an inhibition circle against \textit{S. aureus} (HH et al., unpublished results), and Batch #3: 71 strains that were previously found in our laboratory to exacerbate the \textit{Staphylococcal} infection in silkworms (AM et al., unpublished results). To prepare acetone extracts, 20 mL of 4-day culture (YME broth) was mixed with 20 mL of acetone, then the supernatant was dried and dissolved in 2 mL of water. In the control group, the silkworm was injected with saline (50 µL/larva) and injected with 50 µL of a 1000-fold diluted \textit{S. aureus} (MSSA1 strain) overnight culture. The number of surviving silkworms was measured 48 hours after the injection, and those whose survival rate was lower than that of the control group were judged to have immunosuppressive effect. The samples that showed immunosuppressive effects were further examined to see if they killed the silkworm \textit{per se}, and if true those samples were excluded from the hit samples because it may contain toxic substances that is not suitable for further preclinical development.

3. Results

3.1. Starvation stress increases susceptibility of silkworm to infection

The susceptibility of starvation-stressed silkworms to \textit{S. aureus} infection increased with the duration of starvation, and the LD\(_{50}\) value of \textit{S. aureus} MSSA1 strain was up to 20-fold lower (i.e., silkworms being more susceptible) for 3-day starvation conditions than control group (Figure 1a. Estimated LD\(_{50}\) values from logistic regression: Control, 0.74 ± 0.13 \([\times 10^{7} CFU/larva]\); 1-day starvation, 0.34 ± 0.03 \([\times 10^{7} CFU/larva]\); 2-day starvation, 0.072 ± 0.033 \([\times 10^{5} CFU/larva]\); 3-day starvation, 0.043 ± 0.004 \([\times 10^{5} CFU/larva]\)).

3.2. \textit{Cryptococcal} infection increases susceptibility of silkworm to \textit{S. aureus} infection

The LD\(_{50}\) value of \textit{S. aureus} MSSA1 strain was
3.5. Brief search for of immunosuppressive agents from a soil bacterial library

Among 193 soil bacterial strains, 29 strains showed immunosuppressive effects, and the culture supernatants of 24 of these strains were not toxic to silkworms when administered alone. The above 24 soil bacterial strains were each from batch 1 (6 out of 70 strains), batch 2 (8 out of 52 strains), or batch 3 (10 out of 71 strains). The detailed information for each batch is described in the Methods section.

4. Discussion

In the present study, we showed that (1) high temperature stress, (2) starvation stress, (3) Cryptococcal infection, and (4) administration of betamethasone, an anti-inflammatory steroid used in humans, increased the susceptibility of silkworms to infection by S. aureus. These results suggest that the various stressors suppress the immune function of the silkworm, which is consistent with the immunosuppressive responses to stressors that are found in a range of species (18). The susceptibility of silkworm as measured by the LD$_{50}$ value of S. aureus MSSA1 strain was increased by more than 45 times smaller in silkworms infected with a sublethal dose of C. neoformans than control silkworms (Figure 1b. Estimated LD$_{50}$ values from logistic regression: Control, 3.5 ± 0.1 [×10$^7$ CFU/larva]; Cryptococcus pre-infected, < 0.078 [×10$^7$ CFU/larva]).

3.3. Thermal stress increases susceptibility of silkworm to S. aureus infection

The LD$_{50}$ value of S. aureus MSSA1 strain in the silkworm reared at high temperature (37°C) was about 100 times smaller than that in the silkworm reared at normal temperature (27°C) (Figure 1c. Estimated LD$_{50}$ values from logistic regression: 27°C infection, 1.6 ± 0.1 [×10$^7$ CFU/larva]; 37°C infection, 0.012 ± 0.013 [×10$^7$ CFU/larva]).

3.4. Increased susceptibility to infection in silkworms treated with betamethasone

The LD$_{50}$ value of S. aureus MSSA1 strain for silkworms treated with betamethasone were about 100 times smaller than that for silkworms in the control group (10% DMSO-injected group) (Figure 1d. Estimated LD$_{50}$ values from logistic regression: untreated group, 3.0 ± 0.4 [×10$^7$ CFU/larva]; betamethasone treated group, 0.021 ± 0.00001 [×10$^7$ CFU/larva]).

Figure 1. Increased susceptibility to bacterial infections due to high temperature, starvation, cryptococcal infection stress, and immunosuppressive steroids. Dose-response curves of S. aureus infection are shown in the panels. In all four panels (a-d), the x-axis represents the infected dose (× 50×10$^8$ CFU per larva) and the y-axis represents the survival rate of silkworms 2 days after MSSA1 infection. (a) Starvation stress. Black circles indicate the untreated silkworm group. Red circles indicate the starvation groups: 1-day starvation (lightest red), 2-day starvation (middle red), and 3-day starvation (darkest red). (b) Cryptococcus infection stress. Black circles indicate the untreated silkworm group. Red circles indicate groups infected with sub-lethal doses of Cryptococcus prior to MSSA1 infection. (c) Temperature stress. Black circles indicate the group infected at 27°C. Red circles indicate the group infected at 37°C. (d) Effect of immunosuppressive steroid (betamethasone). Black circles indicate the untreated (vehicle injection) silkworm group. Red circles indicate the betamethasone treated group.
mechanism the four stresses suppress the immune function of the silkworm.

Since betamethasone, which shows immunosuppressive effects in human, also showed immunosuppressive effects in silkworm, it may be possible to use silkworm for screening of immunosuppressive agents. The screening of immunosuppressive agents using silkworms has the advantage that the pharmacokinetics and toxicity (ADMET) of candidate compounds can be considered in the early stage of screening, unlike in vitro screening systems. As demonstrated in this paper, 24 out of 193 soil bacterial samples showed immunosuppressive effects in the silkworm and are a good candidate for further development. The molecular properties, such that the active substance was extracted with 50% acetone, facilitate further investigation for the 24 candidates; purification and structural analyses for each substance is the next step of this study. Also, in future, the development of a method to remove or inactivate immunosuppressive agents from soil bacterial samples will improve the efficiency of antibiotics screening using the silkworm system.

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