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Antibacterial immune response and biological control of agricultural insect pests

Odporność przeciwbakteryjna a biologiczna metoda zwalczania owadów szkodników upraw

Insects contain all the components to sustain life and reproduction in their ecological niches contaminated with predators, pathogens and parasites. Selective pressure during evolution resulted in the development of protective mechanisms in insects against microbial invaders and parasites (7). The survival of an insect depends on the successful defence of the body against microbial invasion. The first line of defense that insects have against pathogens is physical and biochemical – the body coverings and anatomical and physiological structures of the midgut (2). Once this barrier is breached, a complex immune reactions develop that rapidly eliminate a wide range of microorganisms from the insect body cavity. The best characterized aspect of this response are haemocyte-mediated defense reactions (15, 18) and the synthesis of antimicrobial peptides and/or proteins in the fat body (3, 9) and certain type of haemocytes (19). The antimicrobial response occurs after the action of stress factors disturbing an insect body integrity or after microbial challenge. When a microorganism or parasite is entered in the haemocoel, the haemocytes respond to the challenge by phagocytosis (16) nodule formation or encapsulation (6). The immune proteins released into the haemolymph provide a broad spectrum protection against a range of bacteria and fungi. The best biochemically defined native immune molecules are haemolymph lysozyme (13), lectins (14), complement-like activity (1), phenoloxidase system (17) and such inducible antibacterial entities as cecropin-family peptides (3) and small proteins of attacin and attacin-like type (8). Viruses, bacteria, fungi and other biologically active ingredients of microbial insecticides could provoke an immune response in pest insects and by this way the inducible immune system may minimize the effectiveness of microbial pesticides used in biological and integrated control of pest insects (10, 11, 12). Obviously, fortuitous biological insect pest suppression can be achieved not only by the change movement of exotic beneficial organisms to new areas and new pests, predators and pathogens that pest population suppression eventually results but also by a modulation of the immune responses in agricultural and forest insect pests.

It is possible that inhibitors of immune mRNA and rybosomal protein synthesis, antimetabolites and cytostatics have a functional importance in the suppression of the inducible non-self response system in insects, allowing thereby the multiplication of pathogens in blood that finally kill insects due to induced bacterial septicaemias (10, 11). Accordingly, apart from a tremendous progress made recently in the classic methods of biological control for still increasing number of insect pests, the modulation of the insect immune system creates a rational background for the development of new alternative and ecologically safety techniques for insect pest suppression.

MATERIALS AND METHODS

INSECT MODEL SYSTEM, VACCINATION PROCEDURE, INHIBITORS AND THEIR DOSAGES

The greater wax moth (*Galleria mellonella: Lepidoptera*) young pupae (2-3 days old) were used as a convenient model system to study the modulation of insect antibacterial cell-free immune response by immunosuppressive agents used. The target insects were cultivated to pupal stage on dark honey drawn combs at 29°C and 70% relative humidity under total darkness. The immune response was induced by intrahaemocoelic infection of the *Galleria* with *Enterobacter cloacae* strain 12, a nalidixic acid-resistant mutant (4). Using a Hamilton micrometer syringe, insects were immunized by injecting approximately 2.7 x 104 bacteria into the thorax. Inhibitors were given at the time of injection of the immunizing bacteria (at a lag phase of immune response induction). A variety of chemicals known to be suppressive agents to mammalian immunity were used at non-toxic doses to depress insect immune response: actinomycin D, cycloheximide, amethopterin (in a form of methrotrexate natrium, Werfft-Chemie, Wien Lederle Labs Div.), cyclophosphamide and hydrocortisone hemisuccinatum (Polfa, Poland).

SAMPLINGS OF HAEMOLYMPH AND ANTIBACTERIAL ASSAYS

Haemolymph for antibacterial assays was collected within 24 hour post-immunization. The insect blood was taken up in *Galleria* and pipetted into ice-cooled Eppendorf tubes containing sterile water and a trace of phenylthiourea to prevent the melanization of the blood. However, the inhibitor of prophenoloxidase activity was omitted in haemolymph samples used to bioassay the lysozyme concentration because of its inhibitory effect on lysozyme activity.

Antibacterial activities of each haemolymph sample, with or without prior treatment with the immunosuppressor, were determined by a conventional cup agar diffusion assay procedure, using either viable log phase cells of *Escherichia coli* D31 (indicator microorganism for antibacterial assay of cecropin-family peptides activity) or freeze-dried cells of *Micrococcus luteus* (detection of lysozyme activity). The inhibitory effects of an immunosuppressive agent that decreases or totally depresses the antibacterial activity in insect haemolymph were indicated by the reduction or total disappearance of the zone lysis of either *E. coli* (inhibition for cecropin-like response) or *M. luteus* (inhibition for lysozyme immune response) around the well.

Bactericidal activity of cecropin peptides was routinely assayed by measuring zones of growth inhibition in thin agar layers with *E. coli* D31 where the wells were filled with *Galleria* haemolymph to be assayed (5). Assay plates were prepared by spreading 10 ml of soft (0.7%) agar medium in sterile

100 mm glass Petri dishes. The agar medium contained nutrient broth with streptomycin sulfate at a concentration of 100 µg/ml, about 3 x 10^5 cells of an indicator bacterium, and a few crystals of phenylthiourea. Inhibition zones around the wells were recorded after 36 hours incubation of assay plates at 28°C.

A specific activity of lysozyme (EC.3.2.1.17; endo-β-(1-4)-N-acetylmuramide-glycanohydrolase) was quantified by lytic zone assay as determined by Mohrig and Messner (13), using freeze-dried *Micrococcus* cells (Sigma) at a concentration of 1 mg/ml of 0.066 Sörensen buffer (pH 6.4). Various dilutions of chicken egg white lysozyme were used as a standard. Diameters of the lytic zones were measured after incubation of the plates at 28°C for 24 hours. The insect lysozyme activity expressed in the term of the egg white lysozyme activity (EC.3.2.1.17) is given in equivalents to µg/ml of chicken lysozyme.

RESULTS

The practice of various biological methods of insect pest suppression has gained acceptance not long ago. The most prominent and successful in biological insect pest suppression is the direct use of pathogenic microorganisms, parasitoids and predators to reduce or regulate insect pest populations to subeconomic levels. The techniques modulating the insect immune response and, by this way, reducing the host resistance to entomopathogens and/or to their toxic products are the most promising new tools for combating insect pests. Obviously, the immunomodulators under consideration function as chemical messengers at intraorganismic level. The internal environment of the insect pest is regulated by the immune system. The basic principle involved in using various metabolic inhibitors of insect immune system for pest suppression is the fact that in individuals of impaired immune system develop bacterial septicaemias, leading to high mortality.

Several metabolic inhibitors differing by their modes of action on mammalian immunity affect at various range the cell-free immune response in the greater wax moth *Galleria mellonella* (*Lepidoptera*) immunized by bacterial challenge. As can be seen in Figure 1, the immunizing bacterium *Enterobacter cloacae* induced the antibacterial response in *G. mellonella*. The haemolymph of non-immunized insects normally contained low level of lysozyme which increased drastically following infection of the insect body cavity with this non-pathogenic bacterium. No bactericidal activity of cecropin-family peptides was noticed in the native *Galleria*, but the antibacterial activity of these immune principles became evident after bacterial inoculation.

The expression of insect humoral immune response was constitutively suppressed by metabolic inhibitors (actinomycin D, cycloheximide, amethopterin cyclophosphamide and hydrocortisone) given intrahaemocoelically at an early stage of induced immune response (Fig. 1), but the modes of action of these inhibitors

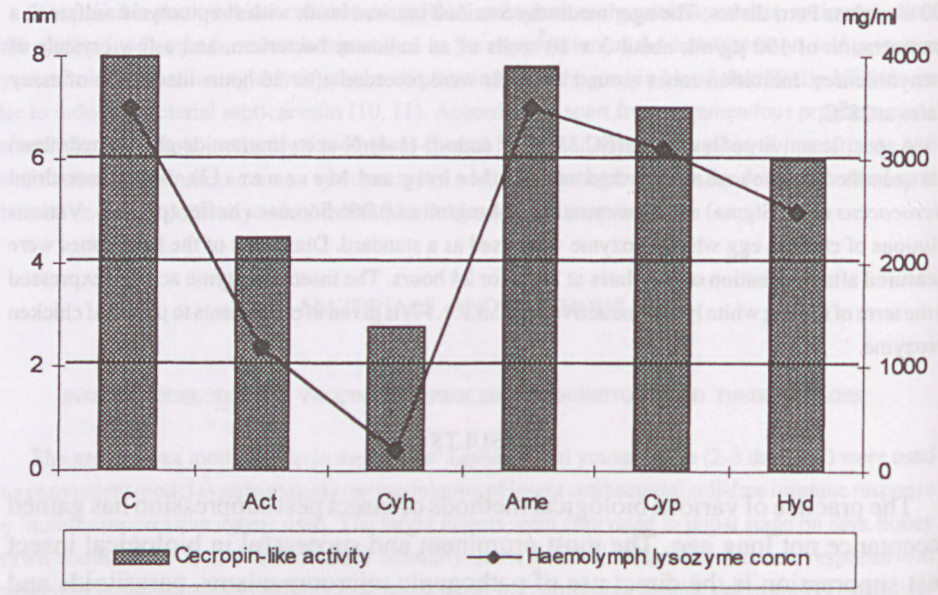

Fig.1. Suppression of cell-free immune response in the greater wax moth (*Galleria mellonella*) by antibiotics known to inhibit mRNA or ribosomal protein synthesis of eukaryotic cells, diverse cytostatics and other metabolic inhibitors; C – controls; Act – actinomycin D; Cyh – cycloheximide; Ame – amethopterin; Cyp – cyclophosphamide, Hyd – hydrocortisone

on the insect non-self response system still requires elucidation. Since the inducible defence system requires nucleic acid and ribosomal protein synthesis the expression of insect immunity is prevented by actinomycin D and cycloheximide. Among cytostatics or antimetabolites other than antibiotics, hydrocortisone evidently depressed the expression of antibacterial cell-free immunity induced by bacterial infection of the insect body cavity. Amethopterin and cyclophosphamide know to be powerful cytostatics only moderately could depress the cell-free antibacterial response in this target insect.

Actinomycin has been regarded as a specific inhibitor of DNA-dependent RNA polymerase and mRNA transcription in *Eukaryota*. Its effect on protein synthesis is normally explained as a consequence of the decay of mRNA. Since the expression of insect multicomponent cell-free immune system requires the *de novo* synthesis of a specific immune mRNA and proteins of a broad antibacterial activity, this specific inhibitor blocked the immune response in *Galleria* though it at a dose of 0.03 µg did not suppress entirely the synthesis of antibacterial proteins. The antibacterial activity of blood lysozyme and cecropin peptides activity in *G. mellonella* was, however, reduced remarkably when compared with lysozyme and cecropin activity in insect given the immunizing bacteria but not treated with actinomycin D.

Cycloheximide specifically inhibits protein synthesis by blocking the translocation reaction on 80S ribosomes. It blocks the elongation phase of protein synthesis on ribosomes by ejecting an old tRNA molecule and resetting the ribosome so that the next aminoacyl-tRNA molecule cannot bind. This specific inhibitor of protein synthesis in mammals and other eukaryotic cells, blocked demonstrably the immune response in the model insect. At a dose of 2.5 μg/individual, it lowered evidently the lysozyme activity below the innate level. Moreover, only few immunized animals have hardly any detectable bactericidal activity of cecropin-like type. Others showed no cecropin antibacterial activity within 24h after treatments. Therefore, this inhibitor of protein synthesis clearly depressed the synthesis of cecropin-family peptides whose activity was reduced to a trace amount in most individuals treated with this specific inhibitor.

Amethopterin, one of the antimetabolites of folic acid synthesis, inhibits the reduction of dihydrofolic acid to tetrahydrofolic acid mediated in cells by the enzyme dihydrofoliate reductase. Consequently, the utilization of tetrafolic acid is prevented. The effects of this dihydrofoliate reductase inhibitor on the cell are reflected in an inhibition of nucleic acid synthesis and cell proliferation. Amethopterin given at a dose of 14.7 μg/insect together with an immunizing bacteria practically did not affect the synthesis of antibacterial immune proteins in *Galleria mellonella*. It is noteworthy that this inhibitor having a powerful cytostatic effect in mammals could demonstrably not depress the expression of insect immune response.

Cyclophosphamide, a bifunctional alkylator with two alkyl chains attached to the nitrogen, disturbs the DNA synthesis by alkylating the purine bases particularly guanine causing cross-linking of the helix. It has a cytostatic effect irrespective whether the cell is dormant or actively proliferating. At a dose of 15 μg per an individual, cyclophosphamide could moderately suppress the immune response in an insect. The hypersynthesis of lysozyme and the *de novo* synthesis of cecropin-like antibacterial peptides was demonstrably reduced in cyclophosphamide-treated animals.

Hydrocortisone, the principal glycocorticoid secreted by the adrenal glands used in the treatment of inflammation, allergies and certain types of cancer, depresses in mammals both humoral immunity and cell-mediated response. In *Galleria mellonella*, this steroid hormone clearly inhibited the synthesis of antibacterial proteins. Hydrocortisone injected at a dose of 12.5 μg depressed the induction of both lysozyme and cecropins in this lepidopterous insect immunized with *E. cloacae*. Briefly, the actual lysozyme titer of immunized *Galleria* decreased evidently in specimens given hydrocortisone. It could not be excluded that hydrocortisone may affect the haemocyte-mediated immune reactions in *Galleria* such as phagocytosis, nodule formation and encapsulation, the principal cellular responses of insect antiinfectious immunity (18).

CONCLUDING COMMENTS

Today, biological insect pest suppression has found a permanent place at the center of the concept of integrated control of pest insects. In some instances it may be complemented by some other techniques, in others, it may itself serve the complementary function. Present advances with the use of microorganisms and predators are evident and encouraging but suppression of immune response to induce bacteriaemias in insects with impaired non-self response system seems to be a new idea that could improve biological techniques of insect pest suppression. The primary goal of biological control is safe for environment, effective and economic reduction of pest population. Obviously, by a variety modes of actions the metabolic inhibitors used in this investigation blocked the expression of the cell-free immune response in *Galleria mellonella,* a model organism commonly used in studies of infection and immunity in insects. It evidently was found that inhibitors depress the *de novo* synthesis of cecropin-family antibacterial peptides and reduce the innate level of haemolymph lysozyme. These immune proteins are thought to participate in the defence system of insects to prevent infection by saprophytic bacteria invading the haemocoel. Experiments thus far conducted fully confirmed this suggestion (12). We observed inducible fatal bacteriaemias in individuals with impaired immunity. In insects treated with metabolic inhibitors, the immunizing bacterium *Enterobacter cloacae* multiplied to a high level in the haemolymph, causing death in nearly 100% of *Galleria* due to *E. cloacae* bacteriaemia.

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STRESZCZENIE

W efekcie ewolucji powstały immunologiczne mechanizmy obronne chroniące owady przez zakażeniem drobnoustrojami i inwazjami pasożytniczymi. Infekcje bakteryjne oraz stres poprzez zaburzenie integralności ciała owada indukują odpowiedź immunologiczną komórkową i humoralną. Również składniki aktywne biologicznych insektycydów, takie jak: wirusy, bakterie, grzyby, stymulują układ odpornościowy owadów, przeciwko którym są stosowane, zmniejszając w ten sposób efektywność preparatu biologicznego wykorzystanego w zintegrowanych metodach zwalczania szkodników.

Patogeny lub produkty przez nie wytwarzane (egzoproteinaza *Bacillus thuringiensis*), podobnie jak liczne inhibitory metaboliczne działające supresyjnie na układ odpornościowy ssaków (antymetabolity kwasu foliowego – aminopteryna, analogi pirymidyn – 5-fluorouracyl, cytostatyki – cyklofosfamid, inhibitory syntezy mRNA i białek rybosomalnych w komórkach organizmów eukariotycznych – aktynomycyna D, cykloheksymid, pestycydy), zaburzają mechanizmy odporności komórkowej i humoralnej. W różnym zakresie hamują one *de novo* syntezę przeciwbakteryjnych peptydów odpornościowych z grupy cekropin, powodują spadek aktywności lizozymu hemolimfy, umożliwiają rozmnożenie się patogena zawartego w biopreparacie i wywołanie posocznicy kończącej się śmiercią owada. Zastosowanie biologicznych i chemicznych immunosupresorów stwarza nowe możliwości dotyczące zintegrowanych metod zwalczania owadów szkodników upraw i lasów.