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Relationship of pesticides to insect cell-free immune response

Wpływ pestycydów na odporność humoralną owadów

Since insects poisoned with chemical pesticides are more susceptible to microbial invasions after application of biological insecticides, the integration of chemical, agronomic and biological methods for suppression of insect pests is now a widely discussed problem. Pesticides represent a large group of chemical classes, and different types of chemicals may disturb the insect non-self response system by a variety of mechanisms. Though much assuring evidence about the effects of insecticides on insect mortality is thus far available, the evaluation of pesticide immunotoxicity has not yet been considered in depth.

Insects employ an arsenal of immune reactions, both cellular and humoral in nature, to combat a diversity of non-self components, including microbial invaders and other injurious agents. Several cell-mediated immune responses such as phagocytosis (8), encapsulation (7) and nodule formation (2) are well documented defence mechanisms in insects. The cell-free immunity is attributable to at least three families of antibacterial proteins active in defences of the moths: to the lysozyme (4, 6) that drastically increases after an infection with bacteria or inoculation bacterial products (for example, LPS of Gram negative bacteria) into the body cavity, and to inducible immune molecules represented by cecropin-family peptides and attacin-like antibacterial proteins. The expression of this multicomponent cell-free insect immune system requires the *de novo* synthesis of a specific immune mRNA and proteins with antibacterial activity against a wide range of Gram negative and Gram positive bacterial species (5).

Without any doubt, several inhibitors differing by their modes of action on mammalian immunity could affect the immune responses in insects. Pesticides were some of the first ones to be investigated for their immunomodulatory effects in mammals (9, 10, 11). Experiments on laboratory mammals demonstrated varied effects of pesticides on cells, tissues and biological processes related to the

immune system. Relationship of pesticides to insect immune response is poorly understood. Chemical pesticides of various classes are therefore analyzed as toxicants that could specifically impair the insect non-self response system. There are examined the immunotoxic effects of chlorinated hydrocarbon (lindane), organophosphorous pesticide (trichlorfon), carbamate insecticide (carbaryl) and pyrethroid pesticide (deltamethrin) on cell-free immune response of the greater wax moth *Galleria mellonella* and mortality of larvae parasitized with the insect bacterial pathogen *Pseudomonas aeruginosa*.

MATERIAL AND METHODS

TARGET INSECT, IMMUNIZATION AND INHIBITION OF IMMUNE RESPONSE

Seventh instar larvae of the greater wax moth *Galleria mellonella* (*Lepidoptera: Pyralidae*) were used as an insect model system to analyze the modulation of the cell-free immune response in pesticide poisonings. The stock culture of *Galleria* was grown intensively on dark honey drawn combs at 28°C in total darkness. Antibacterial immune response was generated by intrahaemocoelic vaccination with lipopolysaccharide (LPS) of *Pseudomonas aeruginosa*, and haemolymph samples for antibacterial assays were collected within 24 h post-immunization. A group of four different chemical insecticides was used for experimentation: chlorinated hydrocarbon (lindane), organophosphorous pesticide (trichlorfon), carbamate insecticide (carbaryl) and pyrethroid pesticide (deltamethrin). The pesticides were given at non-toxic doses (0.1 x LD_{50}) into larval body cavity together with the immunizing agent. The median lethal dose (LD_{50}) was evaluated by probit mortality analysis of F i n n e y (1) following intracoelomic inoculation of the pesticide. During experiments larvae were kept at 28°C, except for deltamethrin-treated *Galleria* that were maintained at 20°C.

BIOASSAY FOR ANTIBACTERIAL IMMUNE PROTEINS

A specific activity of lysozyme (EC 3.2.1.17) in haemolymph samples from native, immunized and pesticide poisoned larvae was quantified by a conventional agar-diffusion assay technique (6) using freeze-dried *Micrococcus luteus* (Sigma Chemicals Co), a general substrate for C (chicken) type lysozymes. An assay 10 cm Petri plate consisted of 10 ml 0.066 M Sörensen buffer (pH 6.4), 10 mg *Micrococcus* cells, 1% agarose and 0.7 mg of streptomycin sulfate to inhibit the growth of bacterial contaminators. Several known concentrations of the chicken egg white lysozyme(Sigma, three times crystallized) were used as standards, and the activity of *Galleria* lysozyme was expressed in µg/ml in equivalent to egg white lysozyme.

Antibacterial activity of cecropin family peptides was recorded as the diameter of the inhibition of bacterial zones around the wells (2.7 mm in diameter) in a thin agar layer inoculated with *Escherichia coli* D31 (3). A synthetic peptide of cecropin B (Sigma) *Hyalophora cecropia* was used as a standard, and the cecropin-like activity in insect haemolymph was expressed in µg/ml. Each thin-layer agar plate contained 8.0 ml of a soft (0.7%) nutrient agar inoculated with about 10^5 log phase cells of the bacterial indicator, 800 µg of streptomycin sulfate and a trace of phenylthiourea to prevent the melanization of insect blood due to phenoloxidase activity.

The suppression of cell-free immune response of lysozyme and cecropin-like type in pesticide-treated *Galleria* was indicated by the reduction or a total disappearance of the zone lysis around the well in the assay plates.

PROTECTIVE IMMUNITY AGAINST INSECT BACTERIAL PATHOGEN

The protection (100 minus percent of mortality) was calculated from the cumulative mortality on day 3 due to *Pseudomonas aeruginosa* septicaemia of immunized and then pesticide-poisoned larvae of *G. mellonella*. Broth cultures of *P. aeruginosa* (24 hours old) were microbiologically standardized by the agar colony count and the infection dose (about 1.5×10^3) was prepared in physiological salt solution for moths (12). The *LPS P. aeruginosa*-immunized larvae but not poisoned with a pesticide were also challenged with a many fold lethal dose of *P. aeruginosa*, a potent insect bacterial parasite.

RESULTS

The innate antibacterial activity of the greater wax moth larvae is attributed to haemolymph lysozyme. The concentration of lysozyme in non-immunized larvae is relatively high (from 70 to 600 µg/ml) comparing to other species of insects. Cecropin-family peptides, the defence antibacterial principles of the moths, are considered to be the major component of the inducible immune proteins of insects. Therefore, naive larvae had no cecropin-like activity in their haemolymphs (Fig. 1)

The antibacterial immune response in larval *Galleria* was generated by intra-haemocoelic inoculation with LPS of *Pseudomonas aeruginosa* at the dose of 2.5µg/insect. The immunization induced hypersynthesis of blood lysozyme: the innate level of lysozyme increased to about 13,000 µg/ml in most individuals. In the immunized larvae, there was noticed the *de novo* synthesis of cecropin-family peptides, and the antibacterial activity of inducible immune proteins was more than 100 µg/ml when expressed in term of the synthetic peptide of cecropin B (Sigma) from *Hyalophora cecropia* (Fig. 1).

The median lethal dose (LD_{50}) for pesticides used to intoxicate of the greater wax moth larvae estimated by the probit analyses of Finney pointed to a high toxicity of deltamethrin (LD_{50}, 0.1 µg) comparing to lindane (LD_{50}, 10.1µg), carbaryl (LD_{50}, 20.7 µg) and trichlorfon (LD_{50}, 31.0 µg).

In *G. mellonella* caterpillars, the pesticides at the dose of 0.1 x LD_{50} inhibited at various range the hypersynthesis of blood lysozyme and synthesis of cecropin--like peptides. In suppression of the cell-free immune response of lysozyme type the most active was carbaryl followed by trichlorfon and lindane but deltamethrinonly slightly could inhibit the hypersynthesis of lysozyme in *Galleria*. As can be seen in Figure 1, the range of inhibition of cecropin-peptides activity was as follows: lindane carbaryl deltamethrin trichlorfon. Since the insects have been

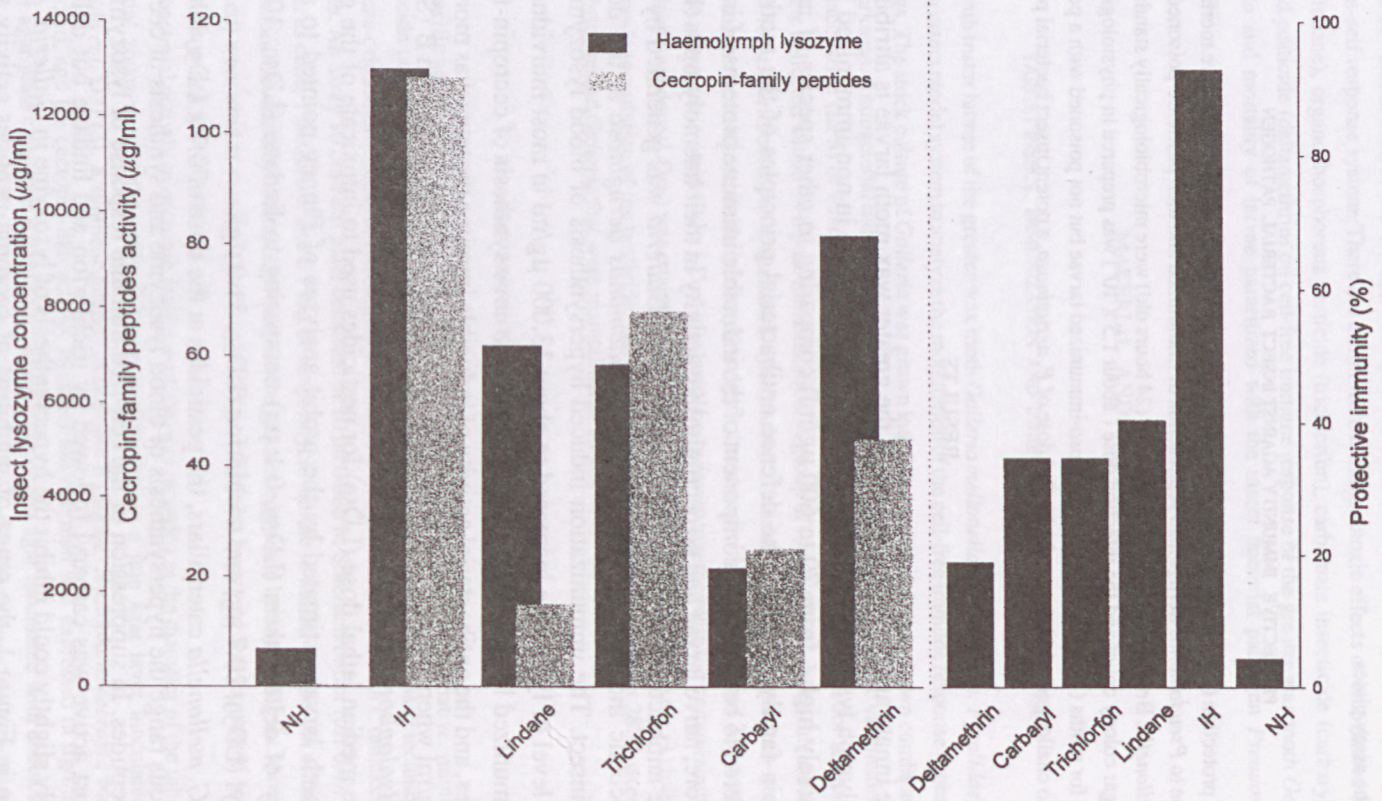

Fig. 1. Relationship between the cell-free immune response and protective immunity against *Pseudomonas aeruginosa* in pesticide poisoned larvae of the greater wax moth *Galleria mellonella*

poisoned with the identical doses (0.1 x LD_{50}) of pesticides used for the experimentation, it is reasonable to conclude that the inhibition of the cell-free immune response in *G. mellonella* by various types of pesticides did not depend on the dosages used but on the insecticide mode of action.

It was found that mortality in non-immunized *Galleria* was nearly 100% after infection with a lethal dose of entomopathogenic bacterium *P. aeruginosa* but almost all immunized specimens survived the bacterial infection (Fig. 1). Obviously, the observed protective immunity is contributed to drastically increased activity in lysozyme and to activity of cecropin-family peptides newly synthesized in vaccinated insects. It is noteworthy that fate of insect bacterial parasite *P. aeruginosa* depended on the expression of the antibacterial immune response in the body cavity of larvae and suppression following pesticide poisonings.

Protection against the bacterial parasite dropped remarkably in the LPS immunized and then pesticide-poisoned *Galleria* larvae. Protective immunity in lindane--treated larvae was 40% but only 20% insects poisoned with deltamethrin survived the lethal dose of *P. aeruginosa.* Similarly, only 30% of insects exposed to toxic action of trichlorfon or carbaryl could survive the bacterial infection (Fig. 1).

Analysis of suppressive action of pesticides on the cell-free antibacterial defence in *Galleria* and protective immunity of insects against *P. aeruginosa* showed only to some extent the relationships between pesticide poisonings, immune responses and mortality of insects. For example, lindane more efficiently depressed the immune response and therefore more efficiently decreased the activity of lysozyme and cecropins, but, when compared, deltamethrin was the most potent inhibitor of protective immunity in vaccinated *Galleria.* In the case of trichlorfon and carbaryl, a significantly reduced protective immunity against *P. aeruginosa* was correlated with depressed antibacterial response in intoxicated larvae of the greater wax moth.

DISCUSSION AND CONCLUDING REMARKS

Integrated pest suppression that involves the compatible utilization of all available forms of pest suppression should be effective and economical in reduction of pest population. The combination of pesticides as modulators of immune response of the pest insects with biological control agents may enhance the success of the integrated methods of pest suppression. Although many aspects of modulation of immune response have been analyzed in great details, some of the fundamental parameters ruling them are still poorly clarified. Moreover, the extent to which chemicals known to be good modulators of mammalian immunity are successful in insects has never been completely and accurately determined.

The categorization of poisoning symptoms is the first step in developing information on the site and mode of action of pesticides. In the case of most insecticides subcategories of compounds based on their poisoning symptoms in mammals are similar to such subcategories established for insect poisoning symptoms. As can be seen from Figure 1, chemical pesticides (lindane, trichlorfon, carbaryl, deltamethrin) at a dose of 0.1 x LD_{50} affect significantly the hypersynthesis of *G. mellonella* lysozyme and the *de novo* synthesis of cecropin-like family peptides in the fat body of LPS *P. aeruginosa*-immunized larvae. The expression of insect cell-free response system was at various range impaired in immunized larvae by pesticide treatments. The antibacterial activities in haemolymph of immunized larvae and then poisoned with insecticides decreased markedly when compared to pesticide-untreated control animals. Accordingly, the protective immunity against *Pseudomonas aeruginosa*, the potential insect bacterial parasite, dropped markedly in pesticide-treated larvae, but it is reasonable to assume that pesticides could also impair haemocytic defenses in *Galleria*. Exposure to pesticides tested herein undoubtedly impairs readily the cell-free antibacterial response in the moth and protective immunity against the parasite *P. aeruginosa*, but there is no evidence that they alter the phagocytosis and other cell-mediated protective mechanisms of the greater wax moth.

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STRESZCZENIE

Organizmy dysponują zespołem odczynów immunologicznych, ściśle współdziałających ze sobą, które chronią przed infekcją. U owadów ilustracją takiego wzajemnego współdziałania jest usuwanie przez lizozym hemolimfy mureinowego zrębu komórki bakteryjnej, pozostawionego po działaniu peptydów odpornościowych z grupy cekropin.

Pestycydy fosforoorganiczne (malation, trichlorfon), węglowodory chlorowane (DDT, lindan), karbaminiany (karbaryl) oraz pyretroidy (deltametrin) w dawkach subletalnych (0,1-0,3 x LD_{50}) w sposób znamienny hamują hipersyntezę lizozymu hemolimfy oraz syntezę cekropinopodobnych (*cecropin-like*) peptydów odpornościowych u *Galleria mellonella* i *Pieris brassicae* uprzednio immunizowanych LPS *Pseudomonas aeruginosa*. W efekcie rozwija się posocznica bakteryjna kończąca się padaniem owadów. U larw z zaburzoną odpowiedzią humoralną działanie ochronne przeciwko *P. aeruginosa* wyraźnie spada, przy czym ten spadek jest w pewnym zakresie skorelowany z nasileniem immunosupresji.

Pestycydy nie stanowią dużego zagrożenia dla układu immunologicznego owada, chociaż niektóre w sposób niespecyficzny mogą zaburzać mechanizmy odporności humoralnej, takie jak aktywność lizozymu względnie polipeptydów odpornościowych z grupy cekropin. Pestycydy mogą też wpływać negatywnie na efektywność fagocytozy i innych komórkowych odczynów obronnych owada. Tak więc stosując kombinacje *Bacillus thuringiensis* lub innych biologicznych insektycydów można będzie zmniejszyć ilość wykorzystywanych chemicznych pestycydów w zwalczaniu szkodników lasów i upraw dzięki wystąpieniu efektu suplementacji.