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RESEARCH REPORT

Immunotoxic action of cyclosporin A on the humoral immune response of *Galleria mellonella* pupae

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Abstract

Cyclosporin A (CsA) was inoculated into the hemocel of pupae in the initial phase of the immune response (at the time of immunization) and in the effector phase of the immune response (within 18 h post immunization). The results obtained indicate suppression of the humoral immune response of pupae after treatment with the antibiotic CsA in both the initial and the efector phase. The immunosuppressant decreased the lysozyme activity against *Micrococcus luteus* and the activity of antibacterial peptides against *Escherichia coli* in the hemolymph within 3 days after injection. The peptide activity decreased more rapidly in comparison to the activity of lysozyme. After 72 h incubation, the reduction in the lysozyme activity was about 55 % in comparison to the activity in immunized insects and only traces of activity against *E. coli* were observed. Differences between the untreated and immunosuppressant-treated insects were statistically significant. The decrease in the lysozyme activity and the antibacterial peptide activity was correlated with loss of protective immunity against *Pseudomonas aeruginosa*.

Key Words: cyclosporin A; immunity; lysozyme activity; peptide activity; *Galleria mellonella*

Introduction

Cyclosporin A (CsA) is a cyclic undecapeptide isolated from the fungi *Tolypocladium inflatum* and *Cylindrocarpon lucidium.* It is a powerful immunosupressive agent used in transplantation immunology (Britton and Palcios, 1982; White and Calne, 1982; Thomson, 1983; Thomson *et al.*, 1984; Weil, 1984; Shevach, 1985). CsA has clinical application in the treatment of autoimmune disorders (Laupacis *et al.*, 1982). In human monocytes and macrophages, CsA induced apoptosis and inhibited neutrophil functions *in vitro*. CsA appeared to be effective in lowering chemotaxis, superoxide anion production and lysozyme release induced by different agonists (Spisani *et al.*, 2001).

Cyclosporins exhibit potent immunosuppressive, antifungal, antiparasitic, antiviral and insecticidal activities. They are able either to kill the infected insect or to incapacitate its immune system (Vilcinskas *et al.*, 1999). Cyclosporins showed insecticidal activity against mosquito larvae (Weiser and Matha, 1988). CsA caused pathological changes in all tissues of *Culex pipiens* larvae, but the targets of CsA were mainly mitochondria, which inflated their cristae, disintegrated and changed into vacuoles (Weiser *et al.*, 1989). It was detected that in *Chironomus riparius* larvae this immunosuppressant inhibited the P-glycoprotein related pump, which was able to remove xenobiotics out of the body fluid (Podsiadlowski *et al.*, 1998).

The wax month *Galleria mellonella*, a laboratory model species, is the subject of much current research on insect immunity (Jiang *et al.*, 2010). The studies in *G. mellonella* revealed that lipophorin, a major insect protein, is the CsA-binding protein (Vilcinskas *et al.*, 1997). CsA added to cultivation medium at sublethal concentrations inhibited phagocytosis of isolated *G. mellonella* plasmatocytes (Vilcinskas *et al.*, 1999). Isolated plasmatocytes incubated with CsA exhibited cytoskeleton alterations. CsA enhanced nodule formation accompanied by melanization in *G. mellonella* larvae when injected at sublethal concentrations and coated on particles.

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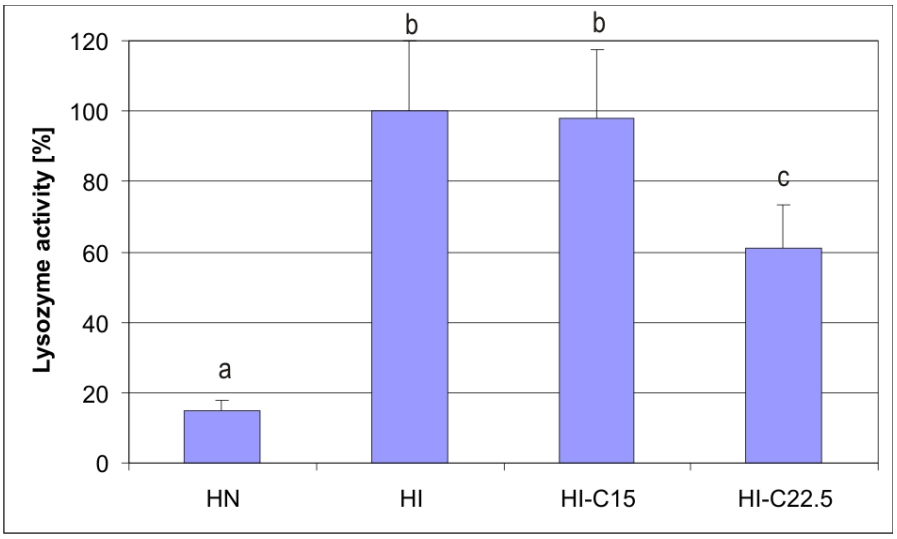

Fig. 1 Lysozyme activity in the hemolymph of *G. mellonella* pupae after immunization and injection with CsA. Samples of pupae hemolymph of: HN-non-immunized control pupae, HI- after immunization with LPS, HI-C15 - after immunization with LPS and injection with CsA at dose 15 µg/insect, HI-C22.5 - after immunization with LPS and injection with CsA at dose 22.5 µg/insect. Bars represent mean ± SD calculated from five independent experiments; b vs a - $p < 0.001$, c vs b - $p < 0.05$.

CsA suppressed the humoral immune response of *G. mellonella* larvae (Fiołka, 2008). CsA moderately decreased the lysozyme activity and significantly decreased the antibacterial activity of peptides against *Escherichia coli*. Immunosuppressive effects were expressed in larvae treated with cyclosporin A both in the initial and the effector phase of the immune response. Insects with an immune response impaired by the CsA action lost their protective immunity to the pathogen *Pseudomonas aeruginosa*.

Since there are no data on the effect of CsA on the humoral immune response in lepidopteran pupae, it was advisable to analyze the action of this immunossuppressant on the antibacterial activity and protective immunity in *G. mellonella* pupae.

Materials and methods

Target insects

Young pupae of the greater wax moth *Galleria mellonella* L. (*Lepidoptera*: *Pyralidae*) were used as an insect model system to study the modulation of the antibacterial cell-free immune response in *G. mellonella* under the influence of CsA. The target animals were incubated on dark honey drawn combs at 28 ^{o}C and 70 % relative humidity under total darkness.

Immunization and immunosuppressant

The induction experiments were performed on 2- to 3-day-old pupae removed from coccons directly before immunization. The humoral antibacterial response was generated by intrahemocelic inoculation of insects with LPS of *Pseudomonas aeruginosa* (Sigma) (39 ng/pupae) using a Hamilton micrometer syringe. Immune hemolymph for antibacterial assays was collected 24 h after immunization. During the experiments, the insects were kept in an incubator at 28 ^{o}C and relative humidity of ~70 %. Control hemolymph was collected from unvaccinated insects. Each experimental group consisted of at least twelve animals. Cyclosporin A (Fluka) was dissolved in 80 % ethanol and 2 µl were injected into the hemocel of the pupae.

Hemolymph collection

After incubation, in each case the hemolymph was collected and tested for antibacterial activity of lysozyme and antibacterial peptides, using the inhibition zone assays in agar plates. The pupae were bled by piercing the region of the thorax and gentle squeezing the insect body. Hemolymph (5 µl volume) was taken up in capillaries and pipetted into ice-cold Eppendorf tubes containing sterile water (dilution 1:5) with a trace of phenylthiourea to prevent melanization of the blood. However, the phenylthiourea was omitted in hemolymph samples used for determination of the lysozyme titres due to its inhibitory effect on the lysozyme activity (Jarosz, 1994). Samples of diluted hemolymph were used immediately for the antibacterial assays.

Antibacterial assay for lysozyme activity

Lysozyme activity was quantified by inhibition zone assays in agar plates as described by Mohrig and Messner (1968), using freeze-dried *Micrococcus luteus* at the concentration of 0.7mg/ml of the assay medium. Each 10 cm Petri dish contained 10 ml of 0.066 M Sörensen buffer (pH 6.4), 100 mg of agarose (Sigma) and 0.7 mg of streptomycin sulphate (Sigma) to inhibit the growth of bacterial contaminators. Wells with diameters of 2.7 mm were punched in the agar layer and then

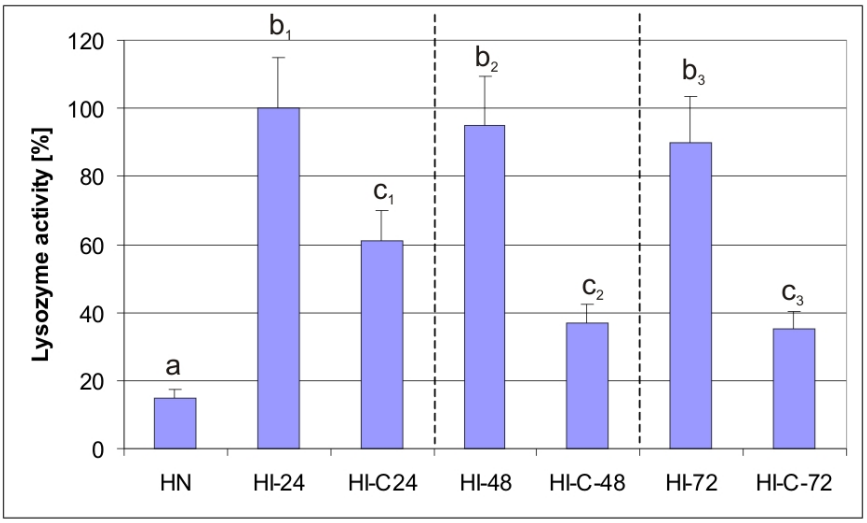

Fig. 2 Lysozyme activity in the hemolymph of *G. mellonella* pupae collected 24, 48 and 72 h after immunization and injection with CsA in the initial phase of the immune response (at the time of immunization). HN-hemolymph of non-immunized, control pupae; HI-24, HI-48, HI-72 - hemolymph of immunized pupae, collected 24, 48, 72 h after immunization; HI-C-24, HI-C-48, HI-C-72 - hemolymph of immunized and injected with CsA pupae, collected 24, 48 and 72 h after immunization. Bars represent mean ± SD calculated from four independent experiments; b vs a - $p < 0.001$, c_1 vs b_1, c_2 vs b_2 and c_3 vs b_3 - $p < 0.05$.

filled with the hemolymph samples to be assayed. Different concentrations of hen egg-white lysozyme (Sigma) were used as standard. Diameters of the lytic zones were measured after incubation of the plates at 28 ^{o}C for 24 h. The antibacterial activity of hemolymph expressed in terms of lysozyme activity (EC.3.2.1.17) is given in equivalents to µg/ml egg-white lysozyme. Maximum activity after immunization was taken as 100 %.

Antibacterial assay for peptide activity

The antibacterial activity of peptides was routinely recorded as the diameter of inhibition zones around the wells (diameter 2.7 mm) in a thin layer of soft (0.7 %) nutrient agar inoculated with the exponential phase cells of *E. coli* D31 (CGSC 5165), a bacterium sensitive to cecropin action (Boman *et al.*, 1974). Pupal hemolymph to be assayed was added into the well (Faye and Wyatt, 1980). The assay plates were prepared by spreading 10 ml of soft agar medium on sterile 10 cm glass Petri dish. Nutrient broth contained streptomycin sulphate (100 µg/ml) and a few crystals of phenylthiourea to inhibit hemolymph melanization due to phenoloxidase activity. Anti-*E. coli* activity in *G. mellonella* hemolymph samples is given in equivalents to µg/ml of the synthetic peptide of cecropin A *Hyalophora cecropia* (Sigma) used as the standard. Inhibition zones were recorded around the wells after 24 h incubation at 28 ^{o}C. Maximum activity after immunization was taken as 100 %.

Protective immunity

The protective immunity (100 minus percent of mortality) against *P. aeruginosa*, a highly virulent bacterium for Lepidoptera (strain H_3), was calculated from the cumulative mortality of *G. mellonella* on day 2 due to *P. aeruginosa* septicaemia (Jarosz, 1994). Overnight broth cultures of the bacterial pathogen were microbiologically standardized by the agar colony count, and a cell suspension of the required density (about $0{,}3x10^2$ in 2 µl for pupae) was prepared in saline W (Weevers, 1966) a physiological salt solution for Lepidoptera. During the 24 h post-immunization, pupae of *G. mellonella* treated with an immunosuppressant were challenged with twelve to fifteen lethal viable cells of the insect bacterial parasite. The insects that had been immunized with the *P. aeruginosa* LPS but not given the immunosuppressant were also challenged with a multifold lethal dose of living cells of *P. aeruginosa*. The onset of the disease was observed then and mortality due to *Pseudomonas* saepticaemia was recorded daily.

Statistical analysis

The Cochran-Cox test was used to determine statistical significance. The differences between statistical parameters were considered significant at $p < 0.05$ and $p < 0.001$.

Results and Discussion

Lysozyme activity

The lysozyme activity in the hemolymph of *G. mellonella* pupae was determined after immunization with *P. aeruginosa* LPS and injection of CsA at different doses (Fig. 1). Afrer immunization, the lysozyme activity was significantly

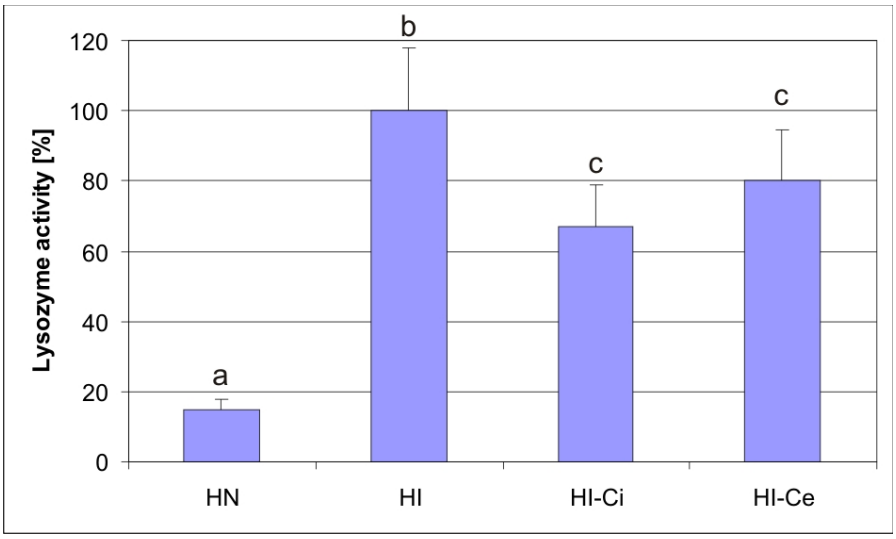

Fig. 3 Lysozyme activity in the hemolymph of *G. mellonella* pupae after immunization and injection with CsA in the initial phase of the immune response (at the time of immunization) and in the effector phase of the immune response (within 18 h post immunization); HN - hemolymph of non-immunized, control pupae; HI - hemolymph of immunized pupae, HI-Ci - hemolymph of immunized and injected with CsA pupae in the initial phase of the immune response, HI-Ce - hemolymph of immunized and injected with CsA pupae within 18 h post immunization in the effector phase of the immune response. Bars represent mean ± SD calculated from four independent experiments; b vs a - $p < 0.001$, c vs b - $p < 0.05$.

increased in comparison to the activity in the control non–immunized insects. Previously, a correlation between lysozyme activity and induced immunity was revealed by Stephens-Chadwick (1970, 1975) and Jarosz (1970, 1985, 1988). In the hemolymph of immunized pupae treated additionally with CsA at a dose of 15 µg/insect, the lysozyme activity was slightly decreased. However, after a higher dose of CsA (22.5 µg/insect), the activity was decreased significantly by about 40 % in comparison to the lysozyme activity in the hemolymph of the pupae that had been immunized but non-treated with immunosuppressant (Fig. 1).

The lysozyme activity in the hemolymph of immunized pupae and those immunized and additionally treated with CsA at a dose of 15 µg/insect was analyzed 24, 48 and 72 h after immunization and inoculation with the antibiotic (Fig. 2). The lysozyme activity was significantly decreased in the hemolymph of the immunized and additionally treated with CsA pupae. Effective suppression of the lysozyme-type cell-free immune response was detectable for 3 days. After 24 h, the reduction in the lysozyme activity was about 39 %, after 48 h 58 % and after 72 h 55 % in comparison to the activity in the immunized insects (Fig. 2).

The immunosuppressant was inoculated into the hemocel of the pupae in the initial phase of the immune response (at the time of immunization) and in the effector phase of the immune response (within 18 h post immunization). After the inoculation with the antibiotic in the initial phase, the lysozyme activity was decreased by about 33 % at time 0, but after injection of CsA within 18 h post immunization, the activity was less inhibited by 20 % in comparison to the immunized pupae (Fig. 3). However, after treatment with CsA in both phases of the immune response, the differences were statistically significant.

CsA at a dose of 22,5 µg/ml effectively decreased the lysozyme activity in the hemolymph of *G. mellonella* pupae, while the larvae of this insect were more sensitive. CsA at a dose of 15 µg/ml had already resulted in significant changes in the lysozyme activity (Fiołka, 2008). The lysozyme activity after inoculation with CsA did not decrease as rapidly in the pupal hemolymph as in the larval hemolymph. The research on larvae showed that the changes in the lysozyme activity were correlated with the changes in the protein level observed after immunoblotting with antibodies against *G. mellonella* lysozyme (Fiołka, 2008).

Antibacterial peptide activity

Antibacterial peptide activity against *E. coli* in the hemolymph of *G. mellonella* pupae was analysed 24, 48 and 72 h after immunization of the pupae and injection of CsA in the initial phase of the immune response. The peptide activity decreased more rapidly in comparison to the activity of lysozyme during 3 days. The activity against *E. coli* evaluated after 24 h was reduced by 50 %, and after 48 h by 53 %, in comparison to the activity in the hemolymph of pupae immunized with LPS but not treated with the antibiotic (Fig. 4). The differences between the untreated insects and those treated with the immunosuppressant were statistically significant. After 72 h incubation, only traces of activity against *E. coli* were observed.

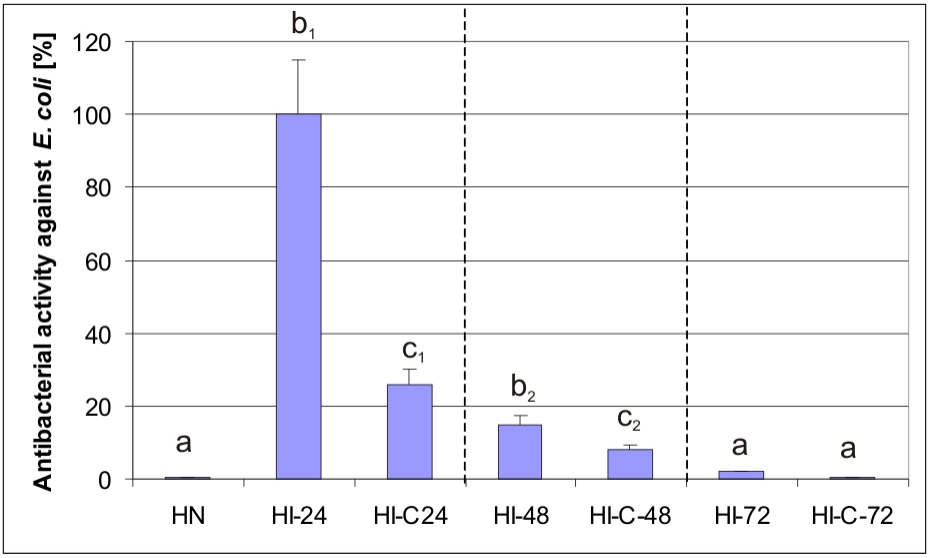

Fig. 4 Antibacterial activity in the hemolymph of *G. mellonella* pupae collected 24, 48 and 72 h after immunization and injection with CsA in the initial phase of the immune response (at the time of immunization). HN-hemolymph of non-immunized, control pupae; HI-24, HI-48, HI-72 - hemolymph of immunized pupae, collected 24, 48, 72 h after immunization; HI-C-24, HI-C-48, HI-C-72 - hemolymph of immunized and injected with CsA pupae, collected 24, 48 and 72 h after immunization. Bars represent mean ± SD calculated from four independent experiments; b vs a - $p < 0.001$, c_1 vs b_1 and c_2 vs b_2 - $p < 0.05$.

Injection of CsA at time 0, immediately after immunization, resulted in a ca.74 % activity decrease in comparison to the activity in the immunologically stimulated pupae. The administration of the antibiotic within 18 h after the immunization with LPS *P. aeruginosa* resulted in a 24 % activity reduction (Fig. 5). In both cases, the differences were statistically significant. The results indicate that inhibition of the antibacterial activity against *E. coli* with CsA in the effector phase was less effective than in the initial phase, as in the case of the lysozyme activity.

Previously, it was observed in larvae that the CsA immunosuppressant administered in the initial phase of the immune response almost completely inhibited the activity against *E. coli*, while in pupae, the suppressive effect was less pronounced. It is known that, in *G. mellonella* larvae, the reduction in the titres of anti-*E. coli* peptide activity after injection of CsA was associated with inhibition of peptide synthesis (Fiołka, 2008). Probably, in the pupae the effect is similar to that observed in the larvae.

Protective immunity

The decrease in the lysozyme activity and antibacterial peptides in *G. mellonella* pupae allowed the enthomopathogenic bacterium to multiply in the insect celomic cavity. Pupae with the immune response impaired by the CsA action lost their protective immunity to the insect pathogen *P. aeruginosa*. In the CsA-treated pupae, the protective immunity diminished to about 70 % of the maximal resistance detected in immunized insects. All the control non-immunized insects died due to *P. aeruginosa* bacteremia (Fig. 6).

However, the *G. mellonella* pupae were more susceptible to infection with a lethal dose of the insect pathogen *P. aeruginosa*. About 30 % fewer pupae than larvae survived infection with the enthomopathogenic bacterium after treatment of insects with CsA. The correlation between the protective immunity against *P. aeruginosa* and the antibacterial activity of hemolyph in *G. mellonella* was observed by Jarosz (1979, 1984b, 1985).

These results indicate suppression of the humoral immune response of pupae after treatment with the antibiotic CsA, both in the initial and the efector phase of immune response. The immunosuppressant decreased the activity of hemolymph against *M. luteus* and *E.coli* during 3 days after injection. The decrease in the lysozyme activity and antibacterial peptide activity was correlated with the protective immunity against *P. aeruginosa*.

In pupae treated with another known immunosuppressant agent, hydrocortisone, reduced titres of the antibacterial peptide activity and a considerably decreased activity of hemolyph lysozyme were found (Jarosz 1994a, 1994b). Hydrocortisone substantially depressed the hemolymph bactericidal activities induced by *Enterobacter cloacae* in two lepidopteran pupae, *G. mellonella* and *Pieris brasicae* (Jarosz, 1994b). The protective immunity dropped in the hydrocortisone-injected pupae of *G.mellonella*, as in the CsA-injected pupae. This suggests that CsA like hydrocortisone may affect the hemolymph in cells that are active in phagocytosis, and exhibits the immunotoxic action on the humoral immune response of *G. mellonella* pupae.

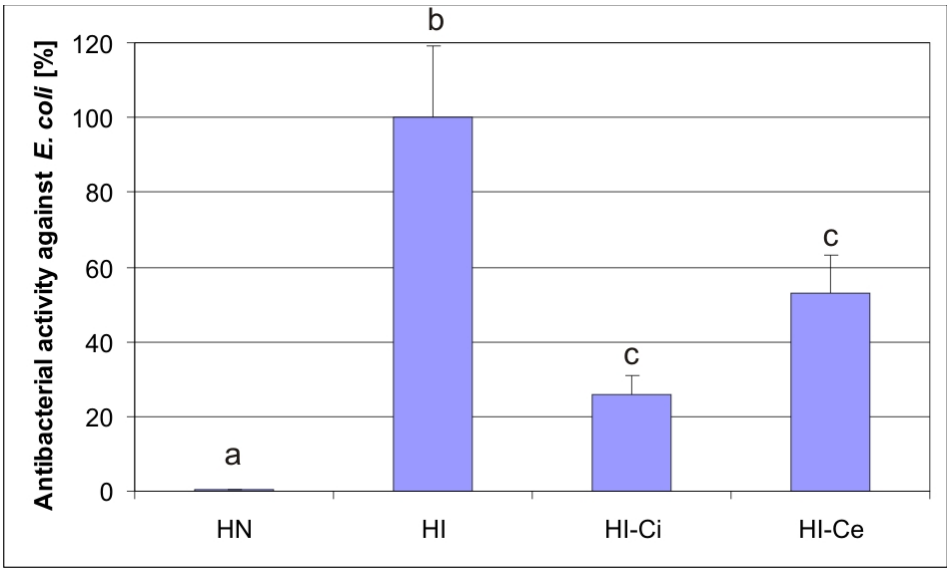

Fig. 5 Antibacterial activity in the hemolymph of *G. mellonella* pupae after immunization and injection with CsA in the initial phase of the immune response (at the time of immunization) and in the effector phase of the immune response (within 18 h post immunization); HN - hemolymph of non-immunized, control pupae; HI - hemolymph of immunized pupae, HI-Ci - hemolymph of immunized and injected with CsA pupae in the initial phase of the immune response, HI-Ce - hemolymph of immunized and injected with CsA pupae within 18 h post immunization in the effector phase of the immune response. Bars represent mean ± SD calculated from four independent experiments; b vs a and c vs b - $p < 0.001$.

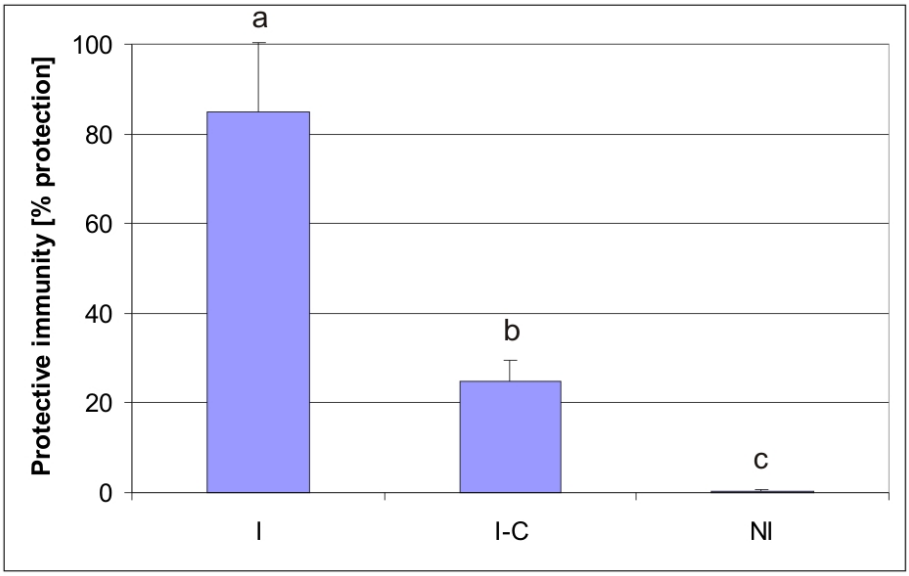

Fig. 6 Protective immunity of *G. mellonella* pupae against *P. aeruginosa* after immunization (I), immunization and injection with CsA (I-C) and of non-immunized pupae (N-I). Bars represent mean ± SD calculated from free independent experiments; b vs a and c vs a - $p < 0.001$.

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