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The antibacterial effect of attacins from the silk moth *Hyalophora cecropia* is directed against the outer membrane of *Escherichia coli*

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The attacins are antibacterial proteins which accumulate in the hemolymph of the giant silk moth, *Hyalophora cecropia*, in response to a bacterial infection. Here we show that the permeability barrier function of the outer membrane is affected shortly after addition of attacin to growing cultures of *Escherichia coli*. Specifically, the penetration through the outer membrane of β-lactam antibiotics, chicken egg white lysozyme and the detergent Triton X-100 was found to be facilitated. The sensitivity of *E. coli* to cecropin B, another antibacterial protein present in the hemolymph of *H. cecropia*, was also found to be increased after treatment with attacin. The results suggest that the target of the attacins in *E. coli* is the outer membrane. Other effects of the attacins which have been observed are likely to be indirect consequences of the alteration in the properties of the outer membrane. These effects include changes in the cell shape, irregular patterns of cell division and lysis. The minimal concentration at which the attacins affected the growth of *E. coli* was 1 and 0.5 μM for the neutral (pI 7) and basic (pI 9) attacins, respectively, which corresponds to <2% of the concentration of the attacins in the hemolymph of infected pupae.

Key words: insect immunity/antibacterial protein/mode of action/bacterial outer membrane/attacin

Introduction

Pupae of the giant silk moth *Hyalophora cecropia* respond to bacterial infection by synthesizing ~15 different proteins, which are secreted into the hemolymph of the insect (reviewed by Boman and Steiner 1981). Among the induced proteins, three different groups have been found to possess antibacterial activity. First, there is the insect lysozyme, which is believed to have functional properties similar to lysozymes from other sources, i.e., to degrade the cell wall, preferentially of Gram-positive bacteria (Powning and Davidson, 1976; Hultmark *et al.*, 1980). In addition there are the cecropins which have been characterized as small basic proteins (mol. wt. 4000), with potent lytic activity against a variety of bacteria (Hultmark *et al.*, 1980; Steiner *et al.*, 1981). Six forms of cecropin, designated cecropins A–F, with small differences in structure but with related activity, have been isolated (Steiner *et al.*, 1981; Hultmark *et al.*, 1982). The third group of antibacterial protein is the attacins (Hultmark *et al.*, 1983), previously referred to as immune protein P5 (Pye and Boman, 1977). Two forms of attacin, one basic (pI 9) and one neutral (pI 7, also referred to as attacin E), have been identified as the major and probably the only native forms in hemolymph of infected pupae (Engström *et al.*, 1984). Both proteins have a mol. wt. of ~20 000, and are present in the hemolymph at high concentrations, ~1 mg/ml at 1 week after infection (Engström *et al.*, 1984). The primary sequences of the two proteins (Engström *et al.*, 1984; Kockum *et al.*, 1984), display an 80% identity and the observed difference in charge between the proteins is explained by a difference in their content of aspartic acid.

Previous studies of the activity of attacins have indicated that the proteins interfere with cell division, and cause lysis of *Escherichia coli*. Activity was found also against two Gram-negative bacteria isolated from the gut of the Chinese oak silk worm, but the proteins were inactive against all other bacteria tested (Hultmark *et al.*, 1983).

Here we describe the mode of action of the two attacins against *E. coli*. Our results indicate that the primary target is the outer membrane of the cell, the initial effect of the attacin being a change in the permeability properties of the outer membrane. After prolonged exposure to attacin physical alterations in the structure of the cell envelope are revealed by electron microscopy. Other effects of the attacin can be explained as secondary effects, which result from the disturbance in the outer membrane structure.

We have found that attacin, at the concentrations found in the infected insect (50–60 μM), affects growth of a variety of bacteria, including Gram-positive species.

Results

Attacin inhibits growth of E. coli

Attacins, isolated from *H. cecropia*, have been shown to inhibit the growth of *E. coli* in conventional antibiotic assays (Hultmark *et al.*, 1983). We have now examined the effect on growing cells of the two native attacins, attacin E (pI 7) and basic attacin (pI 9). When added to growing cultures of *E. coli*, attacin E was found to cause a decrease in the growth rate of the culture, which was first detectable at 1–2 h after addition of attacin. At 3–4 h after addition, growth was completely inhibited (Figure 1A). The minimal antibiotic concentration (MAC), defined as the minimal concentration at which an effect on the growth of the culture could be detected, was 1 μM, against *E. coli* K12 strain D11, and against several different *E. coli* strains tested. The only exception found was *E. coli* D22, an *envA* mutant which, as previously reported (Hultmark *et al.*, 1983), was found to be ~10 times more sensitive than the wild-type to attacin. At concentrations between 1 and 8 μM a decrease in the growth rate was observed but the final density of the cultures was similar to that of control cultures. Total inhibition of growth occurred at attacin E concentrations of 8 μM or higher. The time required for attacin to inhibit growth of *E. coli* was not dependent on the cell density at the start of the experiment, within the range of $5 \times 10^5 - 2 \times 10^8$ cells/ml.

The change in the number of viable cells in cultures treated with attacin E was parallel to the change in cell density, as measured by turbidimetry (Figure 1B). Thus, the attacin E in-

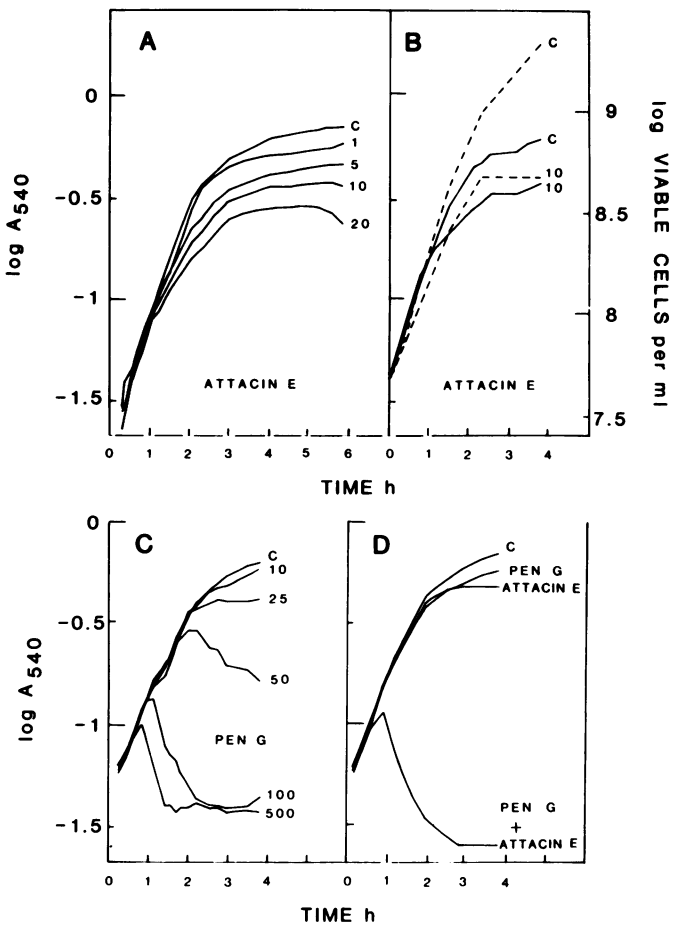

Fig. 1. Effect of attacin, penicillin G and the two compounds combined, on the growth of *E. coli* D11. The cultures were grown in L-broth as described in Materials and methods. Additions of attacin E and penicillin G were done at time 0, the concentrations, in μM for attacin E (**A,B**), and in μg/ml for penicillin G (**C**) were as indicated in the figure. Control cultures, grown without additions, are indicated by the letter c in the figure. In **B** samples were withdrawn from the cultures at 0, 1.5, 2.3 and 3.8 h, and spread on agar plates, containing L-broth, for estimation of the number of viable cells per ml (dashed lines). In **D** the concentration of penicillin G was 10 μg/ml and that of attacin E 4 μM.

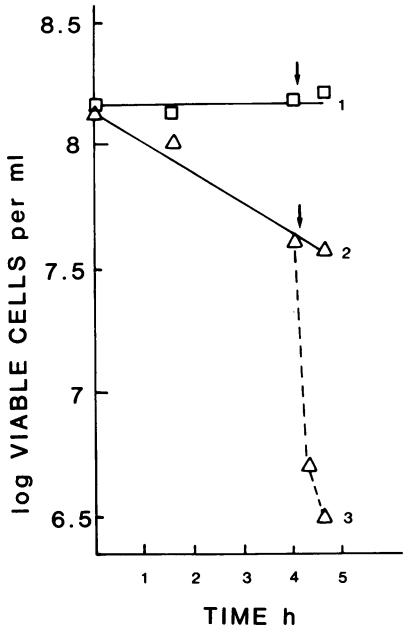

Fig. 2. Effect of attacin on non-growing *E. coli*. *E. coli* D11 was grown in L-broth to a density of 2 x 10^8 cells/ml, pelleted by centrifugation and resuspended in 100 mM potassium phosphate pH 7.0, 1 mM sodium succinate, to approximately the same density. The suspension was pre-incubated on a shaker at 35°C for 45 min before the start of the experiment. The same physical conditions were used during the experiment. At 0 h the suspension was divided into two parts, 1 and 2, and basic attacin (20 μM) was added to 2. At 4 h, suspension 2 was divided into two parts, and Triton X-100 (to 1%) was added to one of these, referred to as 3 in the figure, as well as to suspension 1 (indicated by arrows in the figure). Samples were withdrawn at times indicated in the figure, and spread on agar plates for the estimation of viable counts.

duced inhibition of growth at 4 h after addition was reversible, and the cells maintained viability.

However, prolonged exposure to attacin at concentrations >8 μM resulted in a decrease in cell density, associated with cell lysis. The extent of lysis varied between experiments, possibly as a result of slight differences in the physical treatment of the cells during the experiment. Typically, the rate of lysis was relatively low. At 10 h after addition of attacin, 10–50% of the cells were still viable (data not shown). On prolonged incubation at concentrations of attacin E below 15–20 μM the cultures recovered and grew up to a stationary phase density close to that of the control culture. A likely explanation for the recovery of growth is that the attacin was proteolytically degraded during the incubation and thus the effective concentration of the protein was sufficiently reduced to permit growth of the culture. Degradation of attacin is also the most likely explanation for the unexpectedly high values (20–50 μM) obtained in attempts to determine the minimal inhibitory concentration (MIC) of the protein against *E. coli*. In such experiments *E. coli* cells were inoculated into medium containing attacin and growth was recorded after overnight incubation, the MIC being the minimal concentration at which no growth could be detected.

In experiments where the attacin was removed, at times up to 90 min after addition, either by washing or by addition of trypsin to the culture, the culture continued to grow at the normal rate until the same stationary phase density as that of the control culture was attained. In contrast, removal of the attacin at 120 min, or later, after addition did not abolish its bacteriostatic effect. However, the recovery of growth of such cultures was faster than that of cultures from which the attacin had not been removed.

Our data suggest that the initial effect of attacin E on cultures of *E. coli* is bacteriostatic. To examine the effect of attacin at the cell level, it was added to cells suspended in phosphate buffer, under conditions that did not allow growth of the culture. As shown in Figure 2, basic attacin at 20 μM under these conditions caused a decrease by ~75% in the number of viable cells during a 4 h incubation. In a similar experiment, using attacin E at 10 μM, a 25% decrease in viability was recorded. No change in the turbidity of the attacin-treated suspensions was recorded during the incubation (data not shown). Thus, growth or cell division was not required for attacins to have an effect on bacterial cells.

The basic attacin had effects on the growth of *E. coli* that were qualitatively identical to those of attacin E. However, the specific activity of the basic attacin was approximately two times higher than that of attacin E, as measured in experiments of the kind showed in Figure 1A. Thus the MAC for the basic attacin against *E. coli* strain D11 was ~0.5 μM.

Attacin affects the permeability properties of the outer membrane

To investigate whether the effect of attacin was related to that of other growth inhibitors, we studied the combined effect of attacin and several other antibiotic agents on the growth of *E. coli* cultures. The results showed that treatment with attacin had little or no effect on the sensitivity of the cells towards the following antibiotics: streptomycin, erythromycin, novobiocin, rifampicin, fusidic acid, chloramphenicol and polymyxin B (data not shown).

However, the attacins had a pronounced effect on the activity of penicillin G towards *E. coli* (Figure 1C,D). Under the experimental conditions used (see Materials and methods and legend to Figure 1), the effect of penicillin G on the growth of *E. coli* D11 could be approximately quantified in the concentration range 10–500 μg/ml, with 10 μg/ml being the lowest concentration at which an effect on the growth of the culture could be detected (Figure 1C). At higher concentrations of penicillin G lysis was induced, at times which were dependent on the penicillin G concentration. Addition of attacin E altered the effect of penicillin G on cultures in a quantitative manner (Figure 1D). 1 μM attacin E caused an increase in the effect of penicillin G by a factor of ~2, e.g., 1 μM attacin E added together with penicillin G at a given concentration within the range 5–250 μg/ml gave an effect similar to that of penicillin G alone at twice the concentration. The increase in the effect of penicillin G was approximately proportional to the concentration of attacin E; 2 μM attacin E caused a 4-fold increase, and 4 μM attacin E or higher an 8- to 10-fold increase in the effect of penicillin G, in the concentration ranges 2–100 μg/ml and 1–50 μg/ml, respectively.

In the experiments where the cultures were pre-treated with one of the two compounds, attacin E at 4 μM or penicillin G at 25 μg/ml, and then subjected to the second compound, lysis occurred at 30 min after the addition of the second compound, regardless of the order in which the additions were made. Trypsin, which causes rapid degradation of the attacin, when added to such cultures at times up to ~10 min before lysis, was found to prevent lysis. The treated culture continued to grow at the rate of the control culture grown in the presence of penicillin G only (data not shown).

Similar results were obtained in experiments where ampicillin was used in place of penicillin G. However, no such potentiating effect of the attacins was found on the effect on *E. coli* of two other penicillin analogs; mecillinam and cephalexin (data not shown).

Since the enhanced antibacterial effect of the combined treatment was observed only at concentrations of attacin E at or above the MAC, the effect was not strictly synergistic. In addition, the effect of the combined treatment on the bacteria appeared to be identical to the effect of penicillin G alone at a higher concentration. We interpret these results to mean that attacin induces an enhancement of the penicillin activity against *E. coli*.

The activity of a given penicillin antibiotic against a Gram-negative bacterium is dependent on the concentration of the penicillin in the periplasmic space, which, in turn, is determined by two factors; the rate of passage of the compound through the outer membrane, and the rate of its degradation by periplasmic β-lactamases of the cell (Nikaido, 1980). Since the attacins had no effect on the β-lactamase activity of an *E. coli* extract *in vitro* (performed according to Ross and O'Callaghan, 1975, data not shown), we favour the interpretation that attacin, already within 30 min after addition to cultures, affected the outer membrane of *E. coli*, in such a way as to increase its permeability for penicillin G.

Table I. Effect of Triton X-100 and chicken egg white lysozyme on the survival of *E. coli* D11

Pre-treatment	Treatment	% Surviving cells
1. none	TX-100	100
2. Attacin E	TX-100	<0.01
3. none	Lysozyme	100
4. Attacin E	Lysozyme	45

Bacteria were grown for 4 h as described in the legend to Figure 1, with (2 and 4) or without (1 and 3) the addition of Attacin E to 10 μM (= pre-treatment). Triton X-100 or chicken egg white lysozyme was added to 1% and 200 μg per ml, respectively (= treatment), and 10 min later samples were withdrawn for estimation of the number of viable cells by spreading on agar plates. The numbers of surviving cells are given as % of cells in the cultures after the pre-treatment.

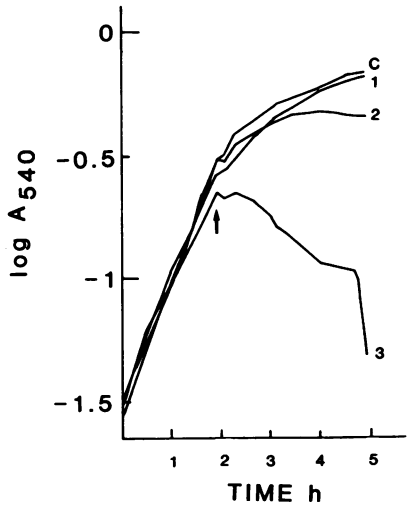

Fig. 3. Effect of cecropin B on cultures of *E. coli*, growing in the presence (3) or absence (1) of basic attacin. Basic attacin (4 μM) was added at time 0 to 2 and 3, cecropin B (5 μg/ml) at 2 h, as indicated by arrow, to 1 and 3. Experimental conditions were otherwise as described in the legend to Figure 1.

To examine further the impact of attacin on the functions of the outer membrane as a permeability barrier, we subjected attacin-treated cells to Triton X-100. Although this non-ionic detergent, at a concentration of 1%, readily dissolved preparations of cytoplasmic membrane of *E. coli*, the survival of an *E. coli* culture was not affected by Triton X-100 (Table I). However, the sensitivity to the detergent of cultures that had grown for 4 h in the presence of attacin, was drastically increased; within 10 min after addition of Triton X-100, the number of viable cells was reduced by four orders of magnitude (Table I). Similarly, cells treated with attacin in phosphate buffer under conditions that did not allow growth of the culture also showed an increased sensitivity to Triton X-100; a 90% decrease in viability occurred within 10 min after the addition of Triton X-100 to a final concentration of 1% (Figure 2). Treatment with Triton X-100 did not affect the viability of the control culture (Figure 2).

Treatment with attacin also caused ~50% of the cells to be sensitive to egg white lysozyme (Table I). This enzyme is inac-

tive against *E. coli*, at the concentration used in the experiment, unless the cells have been subjected to conditions which disrupt the structural integrity of the outer membrane, e.g., by treatment with EDTA.

In addition, growth in the presence of attacin for 2–3 h resulted in an increased sensitivity of cells towards cecropin B. Whereas cecropin B, when added during late log phase at 5 μg/ml, had little effect on the growth of an *E. coli* culture, the same addition to an attacin-treated culture prevented a further increase in cell density, and led to massive lysis of cells (Figure 3).

Discussion

Several lines of evidence suggest that the primary activity of the attacins is directed towards the cell envelope, and specifically the outer membrane of the *E. coli* cell: (i) attacin, at micromolar concentrations, and within 30 min after addition, affects the passage of penicillin through the outer membrane; (ii) longer exposure to attacin causes the cells to be sensitive to the detergent Triton X-100 and to lysozyme, compounds that are normally inactive against *E. coli* due to their inability to penetrate the outer membrane; (iii) changes in the physical structure of the cell envelope are detected after long exposures of the cells to attacin (data not shown).

The change in the permeability of the outer membrane is the first effect of the attacins to be apparent indicating that this structure is most likely the primary target of the attacins. Since the attacins are active against non-growing as well as against growing cells, it is possible that the proteins act primarily to disrupt the structure of already existing outer membrane. However, the concentration of attacin required to affect non-growing cells is considerably higher than that which gives an effect against growing cells. This suggests that the attacins interfere with the synthesis and/or assembly of the outer membrane or some of its components.

Cell lysis was found to be associated with attacin treatment of growing cultures of *E. coli*. Whether lysis is directly or indirectly an effect of the attacin cannot be unambiguously determined. One possibility would be that the apparent bacteriostatic effect of attacin on the growth of cultures is a consequence of a progressively increasing rate of lysis in the culture, leading to a reduction in the growth rate and eventually to a decrease in the cell density of the culture. This would be consistent with the finding that the attacin-treated cells retained the size of exponentially growing cells, and that a fraction of the cells was involved in cell division, also at a stage when the culture was not increasing in cell density. On the other hand, the decrease in the number of viable cells, resulting from attacin treatment of non-growing cells, was not associated with a corresponding decrease in the turbidity of the culture, and thus was probably not correlated with lysis. This indicates that lysis is not the primary consequence of the attacin treatment, but rather an indirect result of the alterations in the cell envelope, occurring only when the cells divide.

Other effects observed, notably the irregular patterns of cell division as well as lesions in the cell envelope observable in the electron microscope (data not shown), are events that occur only after prolonged incubation of cells in the presence of attacin, and are therefore also likely to be secondary effects of a modified cell envelope, and only indirectly caused by attacin.

We cannot explain the existence of two different attacins, since their mode of action against *E. coli* is similar. However, further studies of the activity of the proteins against other bacterial species may reveal differences in their specificity.

The basic attacin and attacin E affect growth of wild-type *E. coli* when added at concentrations >0.5 and 1 μM, and induce lysis of cells at 4 and 8 μM, respectively. This is well below the concentration of the two proteins in the hemolymph of infected pupae, 50 and 60 μM, respectively (Engström *et al.*, 1984). At such concentrations the attacins are active against a variety of bacteria; the growth rates of cultures of *Enterobacter cloacae, Bacillus subtilis, Micrococcus luteus* and *Staphylococcus aureus*, were drastically reduced when the cultures were grown in the presence of basic attacin and attacin E, both proteins at 40 μM (our unpublished observations). Consequently, the attacins, under physiological conditions, are powerful antibiotic proteins, likely to play an important role in the defence of the insect against infecting bacteria.

It is particularly provocative that the attacins, even at concentrations where the effect of the proteins on the growth of cultures is minor, enhance the effect against *E. coli* of cecropin B, which is another antibacterial protein present in the hemolymph of immunized pupae of *H. cecropia*. Similarly, attacin has a potentiating effect on the activity of chicken egg white lysozyme against *E. coli*. The lysozyme present in the hemolymph of *H. cecropia* is believed to be closely related to the egg white enzyme, and we therefore assume that attacin would potentiate the activity also of this protein against *E. coli*. If our conclusions concerning the main target of the attacins are correct, this would mean that the insect is equipped with a three-component defense system, with one protein acting on each of the three main components of the physical barrier protecting the Gram-negative cell; attacin disrupting the outer membrane, lysozyme degrading the cell wall and cecropin attacking the cytoplasmic membrane, all three proteins acting in a concerted way to kill the cell.

Materials and methods

Assay for the determination of antibacterial activity

To minimize the consumption of attacin and to allow simultaneous monitoring of growth of a large number of batch cultures of bacteria, a semi-automated system for data collection and treatment was developed. 200 μl cultures of bacteria, grown in L-broth were contained in microtiter plates (NUNC, Denmark), kept at 35°C on a rotary shaker (Titertek, Flow Laboratories, USA). Each plate has the capacity of 96 cultures. Growth was recorded by monitoring the absorbance at 540 nm in a Titertek Multiskan MC spectrophotometer (Flow Laboratories, USA), which allows the recording of the absorbance of the cultures contained in one plate in ~1 min. The absorbance data were collected by a computer, which was also used for the further handling of the data, to provide growth characteristics and growth curves for the cultures (Figures 1 and 3).

In typical experiments the effect on growth of bacteria of a given compound was assayed by mixing the compound and the growth medium, L-broth. The medium was inoculated with 5 x 10^6 or 10^7 cells taken from a culture in mid-log phase, grown in the same medium, and the absorbance of the cultures was monitored at 5–30 min intervals. For incubations >8 h the plates were sealed with Parafilm in order to prevent excessive evaporation.

The test strain used was D11, an *E. coli* K12 strain which is wild-type in its sensitivity to penicillin-related antibiotics and has the following genotype: F⁻ *trp pro his strA tsx* (Monner *et al.*, 1971).

Preparation of proteins

Basic attacin and attacin E were prepared as described (Engström *et al.*, 1984) from hemolymph of immunized pupae of *H. cecropia*. Cecropin B was a kind gift from H.G. Boman.

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