IB-367 pre-treatment improves the in vivo efficacy of teicoplanin and daptomycin in an animal model of wounds infected with meticillin-resistant Staphylococcus aureus

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Antimicrobial peptides are known as immunomodulators and antibiotic enhancers. We report that administration of an antimicrobial peptide, IB-367, was efficacious in increasing the antimicrobial activity of daptomycin and teicoplanin in a mouse model of wound infection caused by meticillin-resistant Staphylococcus aureus (MRSA). Mice were assigned to seven groups: an IB-367 pre-treated group with no antibiotics given after challenge, two IB-367 pre-treated groups plus daptomycin or teicoplanin given after challenge, two groups treated with daptomycin or teicoplanin only after challenge, and two control groups without infection or that did not receive any treatment. The main outcome measures were quantitative bacterial culture and analysis of natural killer (NK) cytotoxicity and leukocyte phenotype. The wound, established through the panniculus carnosus muscle of mice, was infected by MRSA. Bacterial cultures of mice receiving antibiotics alone showed a -2 log decrease, whilst those for IB-367 plus daptomycin or teicoplanin showed a -4 log decrease. IB-367 plus daptomycin showed the highest efficacy. The higher antimicrobial effect exerted by IB-367 was associated with increased levels of NK cytotoxicity but not of NK cell number. IB-367 increased the number of both CD11b and Gr-1 cells 3 days after MRSA challenge, whereas both of these leukocyte populations were reduced at 10 days after challenge. Our data suggest that a combination of IB-367 with antibiotic exerts a therapeutic effect and may be useful for the management of staphylococcal wounds.

INTRODUCTION

Immune functions are pivotal for defending the body against attack by infection, and therefore can play a pivotal role in the maintenance of health. In particular, natural killer (NK) cells have been shown to play a relevant role in the defence against infectious diseases, being active against viral, bacterial, protozoan and fungal infections (Babizhayev, 2010; De la Fuente & Victor, 2000; Kaminogawa & Nanno, 2004; Messina et al., 1986).

Antimicrobial peptides are cationic and amphipathic molecules isolated from a wide variety of invertebrate and vertebrate animals and from plants (Allaker, 2008; Boman, 2003; Hancock, 2001; Hancock & Scott, 2000).
They have direct antimicrobial activity and the ability to neutralize bacterial endotoxins and to modulate the activities of the innate and adaptive immune systems. Particularly, antimicrobial peptides can confer protection to pathogenic challenge by modulating immune responses (Bowdish et al., 2005). They exhibit a wide range of alternative biological functions that do not target the pathogen directly but rather selectively enhance and/or modulate host defence mechanisms to combat against microbial infections (Boman, 2003; De Smet & Contreras, 2005; Hancock & Scott, 2000; Mookherjee & Hancock, 2007). In fact, several peptides have been shown to strengthen, inhibit or complement cellular functions including apoptosis, chemotaxis, gene transcription, cytokine production, the release of reactive oxygen species, promotion of angiogenesis and wound healing (Auvinet & Rosenstein, 2009; Mookherjee & Hancock, 2007; Salzet, 2002).

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Among these compounds are included protegrins, origin-

ally isolated from porcine leukocytes. These are cysteinerich molecules consisting of 16–18 aa with multiple arginine residues and two disulfide bonds forming a β-sheet (Cho et al., 1998; Giacometti et al., 2003). IB-367 (RGGLCYCRGRFCVGRCONH2), a synthetic protegrin, demonstrated improved bactericidal and fungicidal activity compared with those of native protegrins (Giacometti et al., 2003). The arginine residues make protegrins highly cationic molecules, and this property is primarily implicated in the activity against bacteria.

The selective enhancement of innate immunity by antimicrobial peptides can represent a novel approach as an adjunctive therapy to boost the antimicrobial activity of other compounds. It is important to note that the primary target of these immunomodulatory peptides is the host, and as the effects on the pathogen are exerted indirectly through stimulation of the host’s immunity, the selective pressure for pathogen resistance to the drug is minimal (Auvinet & Rosenstein, 2009; Bowdish et al., 2005; Cenci et al., 2006; Hancock, 2001; Pirofski & Casadevall, 2006).

Wound infections can represent a common indication for the use of antimicrobial therapy. Moreover, the growing bacterial resistance to clinically used antibiotics and lack of alternative effective therapeutic approaches in wound healing pose a pressing problem in patient care, emphasizing the need to develop new approaches for the treatment of infected wounds (Enoch & Harding, 2003; Provinciali et al., 2011; Simonetti et al., 2008). Taking this into account, we hypothesized that a combination of IB-367 pre-treatment and antibiotic treatment post-challenge in an established mouse model of wound staphylococcal infection would act in synergy by IB-367 first stimulating the immune response and the antibiotic then attacking the bacteria.

For this study, we chose two anti-staphylococcal com-
monly used in the clinical setting. Daptomycin is a branched cyclic lipopeptide antibiotic of non-ribosomal origin and is the prototype of the acidic lipopeptide family. It was approved in 2003 for the non-topical treatment of skin-structure infections caused by Gram-positive pathogens, including meticillin-resistant Staphylococcus aureus (MRSA), and in 2006 for the treatment of bacteremia (Steenbergen et al., 2005). Teicoplanin is an old glycopeptide that has come to play a significant role in the therapy of Gram-positive bacterial infections (Pace & Yang, 2006). In particular, it is the choice for empiric therapy of these infections primarily due to its activity against, and the significance of, MRSA.

**METHODS**

**Organism.** The commercially available MRSA ATCC 43300 (Oxoid S.p.A.) was used.

**Animals.** Adult male BALB/c mice weighing 30–50 g were used for all experiments (n=8 per group). The experiments were repeated twice. All animals were housed in individual cages under constant temperature (22 °C) and humidity with a 12 h light/dark cycle, and had access to chow and water ad libitum throughout the study. The environment was temperature and humidity controlled, with lights on and off at 06.30 and 18.30. The study was approved by the animal research ethics committee of INRCA – IRCCS, Ancona, Italy.

**Drugs.** IB-367 was synthesized manually (Faculty of Pharmacy, Medical University of Gdańsk, Poland) by 9-fluorenylmethoxycarbonyl solid-phase chemistry using the protocol as described. The peptide was purified by solid-phase extraction on Kromasil sorbert (C8, 5 μm, 100 Å) (Fields & Noble, 1990; Giacometti et al., 2003). The resulting fractions with purity >97–98% were tested by HPLC. The peptide was analysed by chemical analysis and matrix-assisted laser desorption/ionization (MALDI) mass spectrometry. IB-367 was dissolved in distilled H2O at 20 times the required maximal concentration. Successively, it was diluted in physiological saline. Daptomycin (Novartis) and teicoplanin (Aventis Pharma Spa) were diluted in accordance with the manufacturers’ recommendations yielding 10 mg ml−1 stock solutions. Solutions were made fresh on the day of assay or stored at −80 °C in the dark for short periods. The concentration range assayed for MICs was 0.25–256 mg l−1.

**MIC determination.** MIC was determined according to the procedures outlined by the Clinical and Laboratory Standards Institute (CLSI, 2003) and was taken as the lowest drug concentration at which observable growth was inhibited. For daptomycin, the growth medium was supplemented with Ca2+ to a final concentration of 50 μg ml−1. Experiments were performed in triplicate.

**Preparation of inoculum.** Bacteria were grown in brain–heart infusion broth. When the bacteria were in the exponential phase of growth, the suspension was centrifuged at 1000 g for 15 min, the supernatant discarded and bacteria resuspended and diluted in sterile saline to achieve a concentration of approximately 5 × 107 c.f.u. ml−1.

**Experimental design.** Mice were assigned to seven groups (n=8 per group). Three groups were pre-treated with intraperitoneal IB-367 [1 mg (kg of body weight)−1] at 4 h prior to challenge and on the following 2 days. The study included two control groups without infection or that did not receive any treatment after challenge, an IB-367 pre-treated group with no antibiotics given after challenge, an
IB-367 pre-treated group plus daptomycin (7 mg kg⁻¹) given after challenge, an IB-367 pre-treated group plus teicoplanin (7 mg kg⁻¹) given after challenge, a group only given daptomycin (7 mg kg⁻¹) after challenge and a group only given teicoplanin (7 mg kg⁻¹) after challenge. The main outcome measures were quantitative culture and immunological studies such as immunophenotyping and NK cytotoxicity at 10 days after challenge. The data are presented as means of two different experiments. Immunological studies were carried out on 3 days after challenge in the control untreated group versus the IB-367 pre-treated group.

At time t=0 h, mice were anaesthetized by an intramuscular injection of ketamine (50 mg kg⁻¹) and xylazine (8 mg kg⁻¹) and the hair on the back was shaved and the skin cleansed with 10% povidone/iodine solution. Using a 1.0 × 2.0 cm template, one full-thickness wound was established through the panniculus carnosus muscle on the back subcutaneous tissue of each animal. A small gauze was placed over each wound and inoculated with 5 × 10⁶ c.f.u. MRSA ATCC 43300. The pocket was closed by means of skin clips (Simonetti et al., 2008). This procedure resulted in a local abscess at 24 h. One wound was created per animal. The animals were returned to individual cages and thoroughly examined daily. After 24 h, in control animals the wound was opened, the gauze removed for quantitative bacterial culture and treatment was initiated. Intraperitoneal antibiotic (daptomycin or teicoplanin) was administered daily for 7 days.

Animals were euthanized and a 1 × 2 cm area of skin, including the wound, was excised aseptically. Skin samples were divided into two. One piece was used for histological examination (see below) and the other was homogenized in 1 ml PBS using a stomacher. Quantitation of viable bacteria was performed by culturing serial dilutions (0.1 ml) of a bacterial mixture was then incubated for 30 min in the dark on ice. After incubation, the cells were washed, resuspended in PBS and labelled with mAb. The mixture was then incubated for 30 min in the dark on ice. After incubation, the cells were washed with cold PBS and analysed using an Epics XL flow cytometer (Coulter). A minimum of 10 000 cells, gated by size (forward scatter) and granularity (side scatter), were analysed using the Sistem II software (Coulter).

**Immunophenotyping.** Whole spleens were dissociated into single-cell suspensions of splenocytes using a gentle MACS Dissociator (Miltenyi Biotec) according to the manufacturer’s instructions. The splenocyte suspension was filtered and then stratified on Lympholyte-M (Cederlane Laboratories). After density-gradient centrifugation, viable lymphocytes were twice washed with PBS, counted and suspended in RPMI 1640 with 10% fetal bovine serum. The phenotype of spleen cells was analysed using the following panel of FITC- and phycoerythrin (PE)-labelled mAbs: anti-CD11b (FITC), anti-Gr1 (FITC), anti-CD49b (FITC), anti-CD4 (PE) and anti-CD8 (PE) (all from eBioscience). Briefly, different aliquots of the spleen cells were washed twice with PBS, counted and analysed using an Epics XL flow cytometer (Coulter). A minimum of 10 000 cells, gated by size (forward scatter) and granularity (side scatter), were analysed using the Sistem II software (Coulter).

**NK assay.** A cytotoxic assay was performed using a fluorimetric method, as reported previously (Provinciali et al., 1992). YAC-1 tumour cells were labelled with carb oxyfluorescein diacetate and incubated with effectors spleen cells. Effectors: target cell ratios from 100:1 to 12:5:1 were tested in triplicate. The percentage of specific lysis was calculated as follows: specific lysis = ([Fmed - Fexp]/Fmed) × 100, where F represents the fluorescence of the solubilized cells after the supernatant has been removed, Fmed is the fluorescence from target incubated in medium alone and Fexp is the fluorescence from target incubated with effector cells. Lytic units (LU) per 10⁶ cells were calculated using a computational method: 1 LU corresponds to the number of effector cells required to produce 20% specific lysis.

### Statistical analysis

For efficacy, the outcome measures for comparison of treatments were the number of bacteria in excised tissues and immunological parameters. All results are presented as group means ± SD. Statistical analysis was performed using ANOVA followed by a Student–Newman–Keuls post-hoc test where appropriate. Significance was accepted when the P value was <0.05.

### RESULTS

**Quantitative bacterial culture of excised tissues**

In *in vitro* studies, both antibiotics exhibited MICs of 0.5 mg l⁻¹, whilst IB-367 showed a lower activity with an MIC of 8 mg l⁻¹.

As shown in Table 1, when mice were challenged with MRSA ATCC 43300 and immediately treated with saline (control group), mean bacterial numbers were significantly higher than those recovered from all treatment groups (P<0.05), with the only exception being the IB-367-only pre-treated group. In both antibiotic-only treated groups, a 2 log reduction in counts was found. The group of mice treated with IB-367 plus daptomycin or teicoplanin showed a 4 log reduction in bacterial load compared with the infected control. The most significant reduction in quantitative bacterial culture of excised tissues was seen in mice receiving IB-367 plus daptomycin, with a reduction in bacterial load of 2.7 × 10³ ± 0.3 × 10³ c.f.u. ml⁻¹ (P<0.05).

**NK cytotoxicity and leukocyte phenotype**

As shown in Fig. 1, at both early (3 days; Fig. 1a) and late (10 days; Fig. 1b) time points, NK activity was higher in infected than in uninfected mice; IB-367 increased the NK activity over the levels observed in the respective controls both in uninfected and in infected (P≤0.05) groups both

### Table 1. Quantitative culture of excised tissues after drug administration in staphylococcal wound infection

<table>
<thead>
<tr>
<th>Treatment</th>
<th>C.f.u. ml⁻¹ (mean ± SD)</th>
</tr>
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<tbody>
<tr>
<td>Uninfected</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Infected untreated</td>
<td>8.4 × 10⁷ ± 1.3 × 10⁷</td>
</tr>
<tr>
<td>IB-367 (1 mg kg⁻¹)</td>
<td>5.6 × 10⁸ ± 0.9 × 10⁸*</td>
</tr>
<tr>
<td>Teicoplanin (7 mg kg⁻¹)</td>
<td>7.3 × 10⁷ ± 1.1 × 10⁷*</td>
</tr>
<tr>
<td>Daptomycin (7 mg kg⁻¹)</td>
<td>3.0 × 10⁸ ± 0.4 × 10⁸*</td>
</tr>
<tr>
<td>IB-367 (1 mg kg⁻¹) plus</td>
<td>4.8 × 10⁹ ± 1.1 × 10⁹†</td>
</tr>
<tr>
<td>teicoplanin (7 mg kg⁻¹)</td>
<td>4.8 × 10³ ± 0.3 × 10³†</td>
</tr>
<tr>
<td>IB-367 (1 mg l⁻¹) plus</td>
<td>2.7 × 10³ ± 0.3 × 10³†</td>
</tr>
<tr>
<td>daptomycin (7 mg kg⁻¹)</td>
<td>2.3 × 10⁹ ± 0.3 × 10⁹†</td>
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*All groups pre-treated with intraperitoneal IB-367 and/or treated with intraperitoneal antibiotics showed significant improvement compared with the group without treatment (ANOVA, P<0.05).
†Groups treated with combined peptides and antibiotics showed significant improvement compared with singly treated groups (ANOVA, P<0.05).
at early and late time points. Higher levels of NK cell activity were observed in teicoplanin-treated mice (with or without IB-367) than in control mice at the later time ($P \leq 0.05$; Fig. 1b). Increased but non-significant modulation of NK activity was found in daptomycin-treated animals in comparison with control infected animals (Fig. 1). IB-367 did not significantly modulate leukocyte phenotype in uninfected mice. As shown in Fig. 2(a), an increased percentage of both CD11b and Gr-1 cells was observed in the IB-367 group at the early time compared with the control infected group ($P<0.05$). No significant change of CD49b cells, or CD4 or CD8 T-cells was observed in either group. At the later time point, in mice treated with IB-367 or antibiotics (with or without IB-367), the percentage of CD11b or Gr-1 cells was decreased in comparison with control infected animals ($P \leq 0.05$), whilst no significant change was observed for CD49b, CD4 and CD8 cells (Fig. 2b).

**DISCUSSION**

Staphylococci are ubiquitous environmental bacteria with minimal requirements for survival and a remarkable ability to adapt to a variety of environments. These infections occur mostly in a nosocomial setting, including catheter-related bloodstream infections, ventilator-associated pneumonia, urinary tract infections, gastrointestinal infections and wound infections (Eliopoulos, 2009; Ippolito *et al.*, 2010; Lowy, 2003; Raad *et al.*, 1998). Furthermore, the overall rate of nosocomial *S. aureus* infections has been found to increase rapidly in some patient populations, especially from community-associated MRSA sources. It is well known that many of these infections involve bacteria that are able to develop antibiotic resistance (Breen, 2010; Eliopoulos, 2009; Lowy, 2003; Stevens, 2009) and for this reason the development of novel antimicrobial agents and new therapeutic approaches is required. In this context, our strategy of using the addition of a potent immune enhancer such as an antimicrobial peptide may improve therapeutic efficacy, reduce the carriage rate and increase the cure rate. Apart from efficient antimicrobial activity, antimicrobial peptides also regulate immunity by functioning as immunomodulators. They not only boost the immune response to prevent infection but also suppress other pro-inflammatory responses to avoid uncontrolled inflammation, thereby resulting in homeostasis of innate cellular defences within an organism (Bowdish *et al.*, 2005; Pertinez *et al.*, 2009; Pirofski & Casadevall, 2006). Regulation of the immune system by these immunomodulatory agents could then be proposed as a potential strategy to strengthen host immune responses against infection and to confer protection in vivo against a range of bacterial infections.
The aim of our study was to evaluate the activity of IB-367 as an immunomodulator and immune enhancer. Specifically, we evaluated whether pre-treatment with this peptide in mice resulted in an enhancement of antibiotic efficacy against staphylococcal wound infection. First, we tested the in vitro activity against MRSA. In in vivo studies, we then observed that IB-367 displayed potential antimicrobial benefit with respect to MRSA when used in combination with either teicoplanin or daptomycin. The significant bacterial inhibition determined by the combination of the peptide and the two antibiotics was associated with immunological changes, mainly represented by a modulation of NK cell activity and of CD11b and Gr-1 leukocyte number. In particular, we believe that IB-367, possibly by increasing the activity of NK cells, was able to cause a 1 log reduction in the bacterial cultures and to contribute to the 4 log reduction in the bacterial cultures observed in the IB-367 plus antibiotics groups. The modulation of CD11b and Gr-1 leukocyte number caused by IB-367 may be explained by an increased representation of these cell populations at early time points and by leukocyte migration from the spleen to the site of infection at later times. This immunoregulatory effect of IB-367 may potentiate the direct antibacterial action of antibiotics.

**Fig. 2.** Effect of in vivo treatment with IB-367 and/or teicoplanin or daptomycin on spleen cells phenotype. BALB/c mice were treated with IB-367 and/or antibiotics as reported in Methods and analysed for spleen cells phenotype by flow cytometry at time \( t=3 \) (a) and \( t=10 \) (b) days after challenge with MRSA. Data are reported as means ± sd and are cumulative of two separate in vivo experiments. Differences in phenotype were evaluated by ANOVA followed by a Student–Newman–Keuls post-hoc test where appropriate. *P < 0.05.
Several studies have revealed that antimicrobial peptides can display immunoregulatory activities on immune cells (Auvynet & Rosenstein, 2009; Pirofski & Casadevall, 2006). These activities are extremely diverse and include stimulation of chemotaxis directly and/or through chemokine production, suppression of bacterially induced pro-inflammatory cytokine production, regulation of neutrophil and epithelial cell apoptosis, modulation of cellular differentiation pathways, modulation of dendritic cell activation and differentiation, and promotion of angiogenesis and wound healing.

The role of antimicrobial peptides in wound healing is supported by the observation that several peptides such as human cathelicidins are highly expressed in epidermal keratinocytes in response to injury or infection of the skin (De Smet & Contreras, 2005; Hancock, 2001). In addition, treatment with temporins and LL-37 led to enhanced re-epithelialization of wounds in an animal model. They can selectively stimulate the chemotaxis of phagocytic leukocytes and can elicit the infiltration of neutrophils and selectively stimulate the chemotaxis of phagocytic leukocytes. Several studies have revealed that antimicrobial peptides may influence keratinocyte proliferation or angiogenesis and epidermal hyperplasia, suggesting that peptides may influence keratinocyte proliferation or migration, as well as a higher degree of granulation tissue formation and collagen deposition (Koczulla et al., 2003; Simonetti et al., 2008). In fact, there is little evidence about a direct antibacterial activity of IB-367 and its ability to improve antibiotic efficacy against microbial injury.

Our experiments showed that in vivo pre-treatment with this peptide alone showed significant antimicrobial activity against MRSA when compared with control infected mice. We report for the first time that IB-367 potentiates the antimicrobial effect of teicoplanin and daptomycin, and that this IB-367 action is related, at least in part, to a modulation of the innate immune response. Antibiotics alone showed a comparable antimicrobial efficacy, but their association with IB-367 significantly enhanced the antimicrobial activities over untreated infected mice.

Overall, our findings suggest that IB-367 may play a therapeutic role as an immune enhancer in antibiotic treatment of staphylococci-infected wounds.

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