Vitamin E improves the *in vivo* efficacy of tigecycline and daptomycin in an animal model of wounds infected with meticillin-resistant *Staphylococcus aureus*

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A relevant bacterial load in cutaneous wounds significantly interferes with the normal process of healing. Vitamin E (VE) is a known immunomodulator and immune enhancer. Here, it was shown that administration of VE before infection was effective at increasing the antimicrobial activity of daptomycin (DAP) or tigecycline (TIG) in a mouse model of wound infection caused by meticillin-resistant *Staphylococcus aureus* (MRSA). A wound was established through the panniculus carnosus of mice and inoculated with MRSA. Mice were assigned to six groups: a VE pre-treated group with no antibiotics given after MRSA challenge; two VE pre-treated groups with DAP or TIG given after MRSA challenge; two groups treated with DAP or TIG only after MRSA challenge; and a control group that did not receive any treatment. Mice receiving each antibiotic alone showed a 3 log decrease in the number of c.f.u. recovered compared with the control group, mice treated with VE plus TIG had a 4 log decrease, whilst mice treated with VE plus DAP had the largest decrease in c.f.u. recovered (5 logs). The increased antimicrobial effect seen from treatment with VE plus antibiotics was associated with increased levels of natural killer cell cytotoxicity, with a more pronounced increase in leukocyte populations in mice treated with VE plus DAP. These data suggest that treatment with VE prior to infection and subsequent antibiotic treatment act in synergy.

**INTRODUCTION**

The number of nosocomial infections, including catheter-related bloodstream infections, ventilator-associated pneumonia, urinary tract infections, gastrointestinal infections and wound infections, is increasing (Owens & Stoessel, 2008; Jones, 2010). Gram-positive aerobes are the most common organisms in hospitalized patients with wound infections (Nichols *et al.*, 1999; Elliott & Lambert, 1999; Elsner, 2006; Breen, 2010). *Staphylococcus aureus* is the most common Gram-positive aerobe among these infections, with meticillin-resistant *S. aureus* (MRSA) being the most common pathogen (Elliott & Lambert, 1999; Elsner, 2006; Stevens, 2009; Breen, 2010). The rate of MRSA infection has increased significantly in recent years, especially from community-associated MRSA sources. Glycopeptides represent the first-line agent for MRSA in hospitalized patients, whilst newer agents, such as linezolid, daptomycin (DAP) and tigecycline (TIG), should be reserved for patients who do not respond to or cannot tolerate this antibiotic therapy (Arbeit *et al.*, 2004; Steenbergen *et al.*, 2005; Pace & Yang, 2006; Peterson, 2008; Bouza, 2009; Steenbergen *et al.*, 2009).

Abbreviations: DAP, daptomycin; MRSA, meticillin-resistant *Staphylococcus aureus*; NK, natural killer; TIG, tigecycline; VE, vitamin E.
Immune functions are indispensable for defending the body against attack by infection and therefore play a pivotal role in the maintenance of health (De la Fuente & Victor, 2000; Kaminogawa & Nanno, 2004; Babizhayev, 2010). In particular, natural killer (NK) cells have been shown to play a relevant role in defence against infectious diseases, being active against viral, bacterial, protozoan and fungal infections (Fitzgerald & Lopez, 1986). Food contains various substances that can control the physiological functions of the body, and modulation of immune responses is one of the most important functions (Heinzlerling et al., 1974a; De la Fuente & Victor, 2000; Kaminogawa & Nanno, 2004; Tsalie et al., 2006). Ingestion of food with immune-modulating activities is considered an efficient way to reduce the risk of infection. Vitamins exhibit important immune-modulating functions by entering cells and regulating gene expression (De la Fuente & Victor, 2000; Kaminogawa & Nanno, 2004; Babizhayev, 2010). Vitamin E (VE) is a family of essential micronutrients composed of lipid-soluble tocophersols and tocopherols with strong antioxidant activity. VE nutritional supplementation is believed to be beneficial in ameliorating the effects of diseases such as neurological and cardiovascular disorders, cancer and chronic inflammation (Brigelius-Flohé et al., 2002; Azzi et al., 2003). Several studies have shown that VE improves the inflammatory response of animals against various infections (Tengerdy, 1990; Finch & Turner, 1996; Han et al., 2000). VE stabilizes the membrane of immune cells and enhances the binding of antigen-presenting cells and T cells (Moriguchi & Itoh, 1997). Taking this into account, we hypothesized that a combination of VE pre-treatment and antibiotic treatment post-challenge in an established mouse model of wound infection due to MRSA would act in synergy, with VE first stimulating the immune response and antibiotics then attacking the bacteria. For this study, we chose two new antibiotics. DAP is a branched cyclic lipopeptide antibiotic of non-ribosomal origin and the prototype of the acidic lipopeptide family. It was approved in 2003 for the non-topical treatment of skin-infections caused by Gram-positive pathogens, including MRSA, and in 2006 for the treatment of bacteremia (Arbeit et al., 2004; Steenbergen et al., 2005). TIG is a new glycolcycline antibiotic with an expanded broad spectrum, including inhibition of Gram-positive, Gram-negative, atypical, anaerobic and antibiotic-resistant organisms. Several studies have shown that both of these antibiotics are effective for the treatment of complicated skin and skin-structure infections (Peterson, 2008).

METHODS

Organisms. The commercially available MRSA ATCC 43300 strain was used.

Animals. Adult male BALB/c mice weighing 30–50 g were used for all experiments (n=8 per group). 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 food and water ad libitum. The environment was temperature and humidity controlled, with lights on and off at 6.30 am and 6.30 pm. The study was approved by the animal research ethics committee of the INRCA-IRCCS, Università Politecnica delle Marche, Ancona, Italy.

Drugs. VE (dl-2-tocopheryl acetate) was purchased from Sigma-Aldrich. DAP (Novartis) and TIG (Pfizer) 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 values were determined according to the procedures outlined by the Clinical and Laboratory Standards Institute (CLSI, 2003) and were taken as the lowest drug concentration at which observable growth was inhibited. For DAP, 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 bacteria were in the exponential phase of growth, the suspension was centrifuged at 1000 g for 15 min, the supernatant was discarded and the bacteria were resuspended and diluted in sterile saline to achieve a concentration of ~5×10⁶ c.f.u. ml⁻¹.

Experimental design. Mice were assigned to six groups. Two groups of mice were fed by oral gavage with 60 mg VE (dissolved in corn oil) (kg body weight)⁻¹ at 30 days prior to challenge. VE was administered three times a week. The study comprised a control group that receive only vehicle treatment (corn oil), a VE pre-treated group with no antibiotics given after challenge, a VE pre-treated group plus DAP (7 mg kg⁻¹) given after challenge, a VE pre-treated group plus TIG (2 mg kg⁻¹) given after challenge, a group given only DAP (7 mg kg⁻¹) after challenge and a group given only TIG (2 mg kg⁻¹) after challenge. The main outcome measures were quantitative culture and immunological studies (immunophenotyping and NK cytotoxicity).

At time 0, mice were anaesthetized by 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 1×2 cm template, one full-thickness wound was established through the panniculus carnosus on the back subcutaneous tissue of each animal. A small piece of 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 after infection. One wound was created per animal. The animals were returned to individual cages and examined thoroughly daily. After 24 h, the wound was opened in control animals and the gauze was removed for quantitative bacterial culture. Treatment was then initiated in the groups, with intraperitoneal antibiotic (DAP or TIG) being administered daily for 7 days.

The 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 later) and the other was homogenized in 1 ml PBS using a stomacher. Quantification of viable bacteria was performed by culturing serial dilutions (0.1 ml) of the bacterial suspension on blood agar plates. All plates were incubated at 37 °C for 48 h and evaluated for the presence of bacteria. The organisms were quantified by counting the number of c.f.u. per plate. The limit of detection for this method was ~10 c.f.u. g⁻¹.

Preparation of spleen cells. Spleen tissue was teased through a 60-mesh sieve in Ca²⁺- and Mg²⁺-free PBS solution (Life Technologies). Spleen cells were stratified on Lympholyte-M (Cederlane

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Laboratories) and separated by density-gradient centrifugation at 300 g for 20 min. Cells from the interface of the gradient were washed twice with PBS and then counted and resuspended in RPMI 1640 with 10% FCS (both from Life Technologies).

Immunophenotyping. The phenotype of spleen cells was determined 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). Briefly, different aliquots of the spleen 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 (Beckman Coulter). A minimum of 10,000 cells, gated by size (forward scatter) and granularity (side scatter), were analysed using System II software (Beckman Coulter).

NK assay. A cytotoxic assay was performed using a fluorimetric method, as reported previously (Provinciali et al., 1992). The percentage of specific lysis was calculated as 

\[
\% \text{LYS} = \frac{F_{\text{med}} - F_{\text{exp}}}{F_{\text{med}}} \times 100
\]

where \(F\) represented the fluorescence of the solubilized cells after the supernatant had been removed, \(F_{\text{med}}\) was the fluorescence from the target incubated in medium alone and \(F_{\text{exp}}\) was the fluorescence from the target incubated with effector cells. Lytic units (LU20 per 10⁷ cells) were calculated by using a computational method. One 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 the immunological parameters. All results are presented as group means ± SD. Statistical analysis was performed using analysis of variance (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, DAP and TIG exhibited MICs of 0.5 and 1 mg l⁻¹, respectively. As shown in Table 1, when mice were challenged with MRSA ATCC 43300 and immediately after treatment with saline (control group), mean bacterial numbers were significantly higher than those recovered from the other treatment groups (\(P < 0.05\)), the only exception being the VE-only pre-treated group. In the groups treated with DAP and TIG alone, a 3 log reduction in counts was found. The group of mice treated with VE plus TIG showed a 4 log reduction in bacterial load. The most significant reduction in quantitative bacterial culture of excised tissues was seen in mice receiving VE plus DAP, in which there was a 5 log reduction in counts (\(P < 0.05\)).

NK cytotoxicity

As shown in Fig. 1, the basal values of NK cell activity were lower, although not significantly, in mice challenged with MRSA than in uninfected mice. VE treatment alone did not change NK cytotoxicity in comparison with untreated infected animals. Treatment with VE plus DAP or TIG significantly increased the NK cell activity over the levels observed in VE-only pre-treated mice (\(P < 0.01\)), and treatment with VE plus DAP or TIG induced higher NK cytotoxicity than in the untreated infected group (\(P < 0.05\)).

Table 1. Quantitative culture of excised tissues after administration of VE, DAP and TIG

<table>
<thead>
<tr>
<th>Treatment</th>
<th>c.f.u. ml⁻¹</th>
</tr>
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<tbody>
<tr>
<td>Infected untreated</td>
<td>(7.3 \times 10⁷ \pm 1.3 \times 10⁷)</td>
</tr>
<tr>
<td>VE (60 mg kg⁻¹)</td>
<td>(6.8 \times 10⁷ \pm 1.7 \times 10⁷)</td>
</tr>
<tr>
<td>DAP (7 mg kg⁻¹)</td>
<td>(4.6 \times 10⁶ \pm 0.5 \times 10⁶)</td>
</tr>
<tr>
<td>TIG (2 mg kg⁻¹)</td>
<td>(5.2 \times 10⁴ \pm 0.9 \times 10⁴)</td>
</tr>
<tr>
<td>VE (60 mg kg⁻¹) plus DAP</td>
<td>(3.7 \times 10⁷ \pm 0.4 \times 10⁴)</td>
</tr>
<tr>
<td>(7 mg kg⁻¹)</td>
<td>(9.5 \times 10⁴)</td>
</tr>
<tr>
<td>VE (60 mg kg⁻¹) plus TIG</td>
<td>(8.9 \times 10³ \pm 2.2 \times 10³)</td>
</tr>
<tr>
<td>(2 mg kg⁻¹)</td>
<td>(2.2 \times 10³)</td>
</tr>
</tbody>
</table>

*Groups pre-treated with VE and intraperitoneal antibiotics showed a significant improvement compared with the group without treatment and the group pre-treated with VE only (ANOVA test, \(P < 0.001\)).
†The group pre-treated with VE and DAP showed a significant improvement compared with the two groups treated with single antibiotics (ANOVA test, \(P < 0.001\)).

Phenotype of leukocyte populations

We evaluated the total number of cells and the percentage of several leukocyte populations in the spleen of mice in uninfected animals and in mice challenged with MRSA. The number of spleen cells did not differ significantly
among the groups (data not shown). As shown in Fig. 2, the challenge with MRSA alone increased the percentage of both macrophages (CD11b) and granulocytes (Gr-1); statistical significance was present only for Gr-1+ cells \((P=0.03)\). No significant change in the level of NK cells (CD49b) or CD4+ or CD8+ T cells was observed in infected mice. In mice treated with VE, the percentage of both CD11b+ cells \((P=0.03)\) and Gr-1+ cells \((P<0.02)\) was decreased in comparison with control infected animals, whilst no significant change was observed in the levels of CD49b+, CD4+ and CD8+ cells. The VE plus DAP combination significantly increased the representation of both CD11b+ and Gr-1+ cell populations over the levels of VE-only pre-treated mice. The percentage of CD11b+ and Gr-1+ cells was significantly higher in VE plus DAP-treated than in VE plus TIG-treated animals (Fig. 1).

**DISCUSSION**

Wound-related infections lead to economic loss secondary to patient morbidity and possible mortality. Many of these infections involve *Staphylococcus* species which are capable of developing antibiotic resistance (Lowy, 2003; Elsner, 2006; Stevens, 2009; Breen, 2010). Community-acquired or nosocomial microbial antibiotic resistance is eroding the miracle of antibiotics and jeopardizing both human and animal welfare. With evidence of increased frequency of resistance to glycopeptides, the development of novel therapeutic agents and approaches is required. In this context, our strategy of using the addition of a potent immune enhancer such as VE may improve therapeutic efficacy, reduce the carriage rate and increase the cure rate.

VE was firstly described in 1922 by Evans and Bishop as an essential micronutrient for reproduction in rats.
(Kaminogawa & Nanno, 2004; Tsalie et al., 2006). It is a fat-soluble vitamin, the main function of which is to maintain the integrity of the intracellular membrane by protecting its physical stability and providing a defence against any tissue damage caused by oxidation. It acts through several mechanisms including immunomodulation and an anti-platelet effect. The role of VE in the topical treatment and prevention of skin disorders is well known. Several studies have shown that its application produces skin rehydration with elasticity and resistance improvements, resulting in faster physiological healing and enhanced epithelialization in patients of all ages (Kaminogawa & Nanno, 2004; Babizhayev, 2010; Zampieri et al., 2010).

The aim of our study was to evaluate the activity of VE as an immunomodulator and immune enhancer. Specifically, we evaluated whether the pre-administration of VE in mice resulted in an enhancement of antibiotic efficacy against staphylococcal wound infection. The limitation of this study was that only MRSA was used. Other studies will be useful to back up the data obtained from this study. Our results indicated that VE had a potential antimicrobial benefit with respect to MRSA when used in combination with TIG or DAP. The significant bacterial inhibition resulting from the administration of VE plus TIG or DAP was associated with immunological changes, mainly represented by modulation of NK cell activity and of CD11b+ and Gr-1+ leukocyte number.

Several natural compounds have shown antimicrobial and sensitizing actions against many bacteria, enhancing the action of a specific antibiotic or reversing the natural resistance of specific bacteria to antibiotics (Simões et al., 2008; Coutinho et al., 2009). However, there are only a few studies on a direct antibacterial activity of VE and its ability to improve antibiotic efficacy against microbial injury (Heinzerling et al., 1974a, b; Fang et al., 1990). With some exceptions, most of the animal studies that have investigated the effect of VE in infectious diseases reported a protective effect associated with its immunostimulatory activity (Tvedten et al., 1973; Heinzerling et al., 1974a, b). Meydani et al. (2005) thoroughly investigated the ability of VE to improve immune functions and to confer protection against viral infection, especially in old age, to correct inadequate VE dietary intake risk. Old C57BL mice infected with influenza A virus after 6 weeks of feeding with adequate levels of VE showed significantly higher lung virus titres after infection than young mice under the same conditions (Hayek et al., 1997). Supplementation with high doses of VE induced a significant reduction in lung viral titre with respect to old mice fed on a diet containing an adequate level of VE. In addition, the age-associated decline in NK cell activity was restored by VE in old but not in young mice, demonstrating that high doses of VE significantly enhance influenza virus clearance in aged mice but only modestly affect young mice. VE supplementation was able to reduce the pulmonary titres of influenza virus and to prevent an influenza-mediated decrease in food intake and weight loss (Sheridan & Beck, 2008). In the same study, other antioxidants tested did not have the same effect as VE on viral titre, thus suggesting that, in addition to its antioxidant activity, other mechanisms might be involved in the beneficial effect of VE in infectious diseases. In another study, VE was shown to protect mice against Diplococcus pneumoniae infection (Heinzerling et al., 1974a). In contrast, VE supplementation did not enhance protection against herpesvirus encephalitis in mice (Sheridan & Beck, 2008).

Our experiments showed that in vivo supplementation with VE alone did not exhibit significant antimicrobial activity against MRSA when compared with control infected mice, and, apart from a reduction in CD11b+ and Gr-1+ cells, did not significantly modify either NK cell activity or leukocyte representation. This evidence agrees with that reported in other experimental models performed on young mice. VE has been demonstrated to be effective in enhancing immune responses by itself only in old age, when VE is often reduced because of an inadequate dietary VE intake and immune alterations (immunosenescence) are present (Meydani et al., 2005; Provinciali, 2009).

We have reported here for the first time, to our knowledge, that VE potentiates the antimicrobial effect of TIG and DAP, and that the VE action is related, at least in part, to modulation of the innate immune response. The significant increase in NK activity found in the groups given VE plus TIG or DAP over the cytotoxicity obtained in VE-only pretreated or untreated infected animals suggests an association of the antimicrobial effect with the immune modulation induced by the two combinations.

Antibiotics alone showed a comparable antimicrobial efficacy, but neither TIG nor DAP alone significantly enhanced NK activity compared with untreated infected mice. Furthermore, it was strikingly clear that the combination of VE plus DAP was the most effective treatment, as this group of mice exhibited the lowest bacterial counts, suggesting that the mode of action of this combination might be complementary and clinically beneficial. The greater effectiveness of the VE plus DAP combination could be related to the preferential increase in both CD11b+ and Gr-1+ cells, which was not present in animals treated with VE plus TIG. Further studies will be required to understand better the mechanisms involved in the synergistic interaction between VE and antibiotics.

In conclusion, our data showed that VE can be used clinically in animals as an immune enhancer and in immunoprophylactic combinations with DAP or TIG to inhibit infection. Future studies will be required to evaluate the potential use of VE as an enhancer of antibiotic therapy in humans, and particularly in those populations who are at greater risk of inadequate dietary intake of VE, such as elderly subjects.

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REFERENCES


