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In Vitro Antimicrobial Effect of Encapsulated Vancomycin on Internalized *Staphylococcus aureus* Within Endothelial Cells

Henry Nettey,
Dinesh Haswani and
Martin D'Souza
Department of Pharmaceutical Sciences, Southern School of Pharmacy, Mercer University, Atlanta, GA

Carl Oettinger
Dialysis Clinic Inc., Atlanta, GA

ABSTRACT Vancomycin (VCN) is a glycopeptide antibiotic that is effective in the treatment of gram-positive bacterial infections, but mainly reserved for methicilin resistant *Staphylococcus aureus*. It is, however, ineffective against intracellular bacteria and hence a particulate form of VCN would be required. Bovine serum albumin (BSA) microspheres of VCN with a mean particle size of 5 ± 1.6 μm were used. Human microvascular endothelial cells internalized both *S. aureus* and VCN microspheres in a time and concentration-dependent manner, however, the uptake was inhibited by cytochalasin D. Action of VCN on *S. aureus* in the intracellular microenvironment decreased the bacterial load considerably.

KEYWORDS Endothelial cells, Cytochalasin D, Vancomycin, Microspheres, Bovine serum albumin

INTRODUCTION

The emergence and continued persistence of methicillin resistant *Staphylococcus aureus* (MRSA) is a real concern in the health field. More disturbing is the fact that this organism is developing resistance to the only effective antibiotic against it–vancomycin (VCN). Resistance to VCN is attributed to the overuse by certain health care officials, and in some cases subtherapeutic doses administered to patients for fear of ototoxicity and nephrotoxicity. Bacterial pathogenesis is highly dependent on the ability of organisms to colonize host tissues (Beachey, 1981). Adherence to host cell tissues by *S. aureus* could occur either by binding to host proteins such as fibronectin, or to normal endothelium by specific receptors (Ing et al., 1997). *S. aureus* is generally considered an extracellular pathogen compared to other facultative intracellular organisms such as *Listeria, Salmonella*, and *Mycobacteria* (Bayles et al., 1998). However, adherence to endothelial cells (EC) by *S. aureus* could lead to internalization of bacteria by EC.

Address correspondence to Henry Nettey, 4419 Cross Lane, Decatur, GA 30035; Tel: 678-793-3106; E-mail: hnettey@msn.com; hcn3@cdc.gov

It has been shown by other groups of researchers that, EC can function as nonprofessional phagocytes, and as such take up *S. aureus* (Hamill et al., 1986; Drake & Pang, 1988; Beekhuizen et al., 1997). The consequence of bacterial internalization is not exactly known. Some studies have shown that *S. aureus* does not multiply within EC (Vann & Proctor, 1987), while others have shown that *S. aureus* does replicate within human cardiac valve EC (Lowy et al., 1988) and epithelial cells (Kahl et al., 2000). The survival and replication of *S. aureus* within endothelial cells (where they are protected from host defenses and antibiotics) plays an important role in the frequency and persistence of invasive staphylococcal infections (Qazi et al., 2001).

The use of albumin as a polymer matrix for the encapsulation of various drug types, for example, anticancer drugs (Willmot et al., 1985; Gupta et al., 1986) and steroids (Lee et al., 1981) has been studied extensively. The hydrophilic nature of albumin, however, makes it more efficient in the transport of hydrophilic drugs. Schafer et al. (1994), have shown albumin microspheres to be among the most suitable systems for targeted drug delivery, because they are relatively nontoxic and biodegradable. In order to be taken up by endothelial cells, microspheres need to be 7 μm or less in size. Drug-loaded microspheres can be used effectively to kill intracellular pathogens. It is the aim of this study to expose endothelial cells to VCN-loaded albumin microspheres prepared by the spray dryer method and *S. aureus*, and to evaluate the effect of VCN on the internalized bacteria.

MATERIALS AND METHODS

Bacteria Strains and Culture Conditions

A lyophilized powder of *S. aureus* strain (ATCC 6538) was purchased from ATCC, Bethesda, Maryland. Tripticase Soy Agar (TSA), Tripticase Soy Broth (TSB), culture flasks, petri dishes, and pipettes were purchased from Fisher Scientific Company, Norcross, Georgia. Bacteria was grown overnight in nutrient broth at 37°C, giving a count of 1×10^8 cfu/mL, as determined from a Mcfarland standard curve at OD_{680}. Bacteria were harvested by centrifugation at $2000 \times g$ for 10 min, washed twice in phosphate buffered saline (PBS) and re-suspended in 20 mL of culture media (MCDB131, supplemented EBM media). Serial dilutions of stock bacteria were made (in EBM media) in order to obtain counts of 1×, 10×, and 100× the endothelial cell count. One inoculum of bacteria (100× EC count) was fluorescently labeled with acridine orange.

Cell Culture and Invasion Assay (Qualitative Study)

Human microvascular endothelial cells (HMECs) and culture media (EBM) were obtained from the Centers for Disease Control (CDC). Endothelial cells were seeded in 24-well plates, at 2.0×10^5 cells per well, and allowed to grow at 37°C in 5% CO_2 for 4 days until about 80–100% confluent. Cells in one well were washed and suspended with 1 mL of trysin/EDTA per well. Cell count per well was determined with a hemocytometer and used to determine the bacterial count. Endothelial cells were exposed to labeled bacteria for 3 hr. Bacteria were then washed off twice with PBS. Extracellular bacteria were killed with gentamicin sulfate solution (100 μg/mL), washed off in PBS and infected cells observed under the fluorescent microscope.

Preparation of Labeled VCN Microspheres

VCN-loaded bovine serum albumin (BSA) microspheres were prepared by the spray-drying method. Briefly, a VCN:BSA/1:5 w/w solution was prepared in enough deionized (DI) water to attain a 5% BSA solution. This solution was partially cloudy, but turned clear upon dropwise addition of 1 M HCl with stirring. The solution was spray-dried in a Model 191 Büchi bench-top mini-spray dryer. The inlet temperature was 120°C and the outlet temperature was between 65°C and 70°C. The rate of aspiration was kept at 90%, and the solution flow rate was set at 4%. The vacuum pump pressure was kept between 600 and 800 nL/hr. The dried powder was collected by a cyclone and left to dry in the collection chamber by running dry air through it for 2 hr. Blank BSA microspheres were prepared in the same way except that the solution did not contain VCN. The powders were then stored in closed dram vials at room temperature until ready to use. The microspheres were cross-linked with 4% glutaraldehyde (25% w/v) in 1-butanol for 1 hr.

Cross-linked microspheres were washed twice with butanol, filtered with a 0.45 μm filter and dried at 37°C overnight. For microsphere uptake studies, 250 mg of blank cross-linked BSA microspheres were suspended in 50 mL of butanol and labeled with 1 mL of fluorescamine (0.1 mg/mL in acetone). The microspheres were dispersed in deionized water and the particle size distribution was determined with a laser diffractometer (SpectrexPC-2000). The microsphere particle size range obtained was between 2 and 8 μm, with an average size of 5 ± 1.6 μm. The drug loading of the microspheres was averaged at 18.6% ($n = 3$) with an encapsulation efficiency of 89%.

Uptake of Microspheres by Endothelial Cells

Fluorescent-labeled BSA microspheres were suspended in EBM culture media to obtain a concentration of 1 mg/mL. Microspheres were dispersed by sonication for 5 min, and 1 mL of the suspension was added to HMECs in 24-well plates. At specific time points, the cells were washed twice with PBS, and the uptake of microspheres was determined using a fluorescent microscope.

Cell Culture and Invasion Assay (Quantitative Study)

Endothelial cells were exposed to various concentrations of bacteria in EBM and incubated at 37°C with 5% CO_2. For each concentration of bacteria, experiments were performed in triplicate. Cells were exposed to bacteria for 1 hr, after which time, all wells were washed twice with PBS. Adhered extracellular bacteria were killed by filling all wells with gentamicin sulfate (100 μg/mL) in EBM. After 1 hr of gentamicin treatment, the wells were washed twice with PBS. Endothelial cells were then incubated in 1 mL of fresh media (EBM), and intracellular bacterial count was determined at specific time points. One 24-well plate was used per time point, the zero time being the time immediately after washing off gentamicin solution. At each time point, the media was washed off with PBS and 1 mL of 1% Triton-X100, was added to each well to lyse endothelial cells. The plates were kept at 4°C for 1 hr, after which, serial dilutions of each well were made and plated on solid agar. After 24 hr of incubation at 37°C, bacterial colonies were counted. In a second set of experiments, endothelial cells were initially exposed to 1 μg/mL of cytochalasin D (in EBM) for 2 hr. The wells were washed twice with sterile PBS and exposed to bacterial concentrations as described above.

Effect of VCN Microspheres or Solution on Internalized S. aureus

A suspension of VCN microspheres (40 μg/mL) was prepared in EBM culture media, and kept in an ultrasonic bath for 3 min to disperse microspheres. The same concentration of VCN solution was prepared in culture media. Serial dilutions of *S. aureus* were used to challenge HMECs. Three treatment groups were employed:

Prophylactic Treatment

HMECs were pretreated with 1 mL of VCN microsphere suspension, solution, or plain media (control) for 2 hr; washed twice with PBS, and then exposed to *S. auerus* for 1 hr. Cells were washed twice with PBS and the wells filled with 100 μg/mL of gentamicin sulfate solution for 1 hr to kill extracellular bacteria. The cells were again washed twice with sterile PBS, and 1 mL of 1% Triton-X100 was added to each well for 1 hr at 4°C to lyse HMECs. Serial dilutions of survived intracellular bacteria were plated on TSA plates overnight at 37°C.

Immediate Treatment

In this group, HMECs were exposed to 1 mL of *S. aureus* culture in EBM culture media for 1 hr. The cells were washed twice in sterile PBS and treated with 1 mL of VCN microsphere suspension, solution, or plain media (control) for 2 hr. The cells were washed with PBS, treated with gentamicin and 1% Triton-X100, and plated.

Delayed Treatment

In this group, HMECs, grown to 50% confluency, were exposed to 1 mL of *S. aureus* culture in EBM culture media for 1 hr. The cells were washed twice in sterile PBS and treated with 1 mL of VCN microsphere

suspension, solution, or plain media (control) 4 hr after extracellular bacteria were washed off, and cells incubated in gentamicin sulfate solution. After 2 hr of incubation, the drug was washed off and the cells were incubated in fresh media. At various time points, cells were lysed and the intracellular bacterial count determined.

RESULTS AND DISCUSSIONS

Uptake of Fluorescent-Labeled *S. aureus*

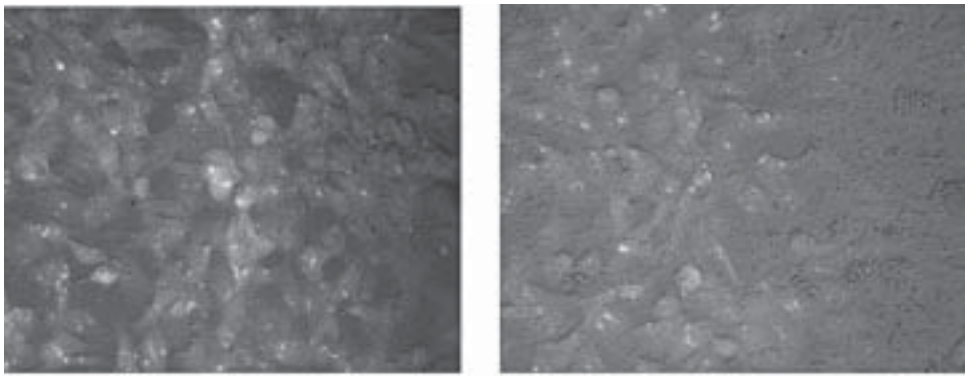
Internalization of *S. aureus* by HMECs was observed under a fluorescent microscope with a digital camera attached, and both connected to a computer. Images were obtained on the terminal after processing by the ISM 50 software. The pictures clearly show that the endothelial cells do take up bacteria, confirming results from previous studies (Hamill et al., 1986; Beekhuizen et al., 1997). The effect of cytochalasin D on *S. aureus* uptake was very profound. Uptake of bacteria by HMECs after cytochalasin D treatment was much less than the untreated cells (Fig. 1), showing that phagocytosis through actin polymerization is a possible mechanism of uptake.

Uptake of Microspheres by Endothelial Cells

Fluorescamine reacts directly with primary amines to form fluorophors. At room temperature, fluorescamine dissolved in a water miscible solvent such as acetone, reacts with and binds to primary amines. In a fraction of a second at room temperature, the reaction is complete and in less than a minute excess reagent is destroyed. Hydrolysis products of fluorescamine are not fluorescent. It is, therefore, expected that only primary amines present on the surface of BSA microspheres will be labeled. Observation of labeled microspheres showed bright green particles of various sizes. Endothelial cells were exposed to labeled microspheres for various lengths of time. Internalization of microspheres by endothelial cells increased with time of exposure, up to about 5 hr. The cells seem to be saturated at that point, and further exposure did not increase uptake to any greater extent (Fig. 2a–c.). Previous studies (Schafer et al., 1994) have shown that albumin microspheres begin to degrade a few hours after uptake and the process is almost completed after 3 days. This process (though unconfirmed) could be ongoing as seen in the figures.

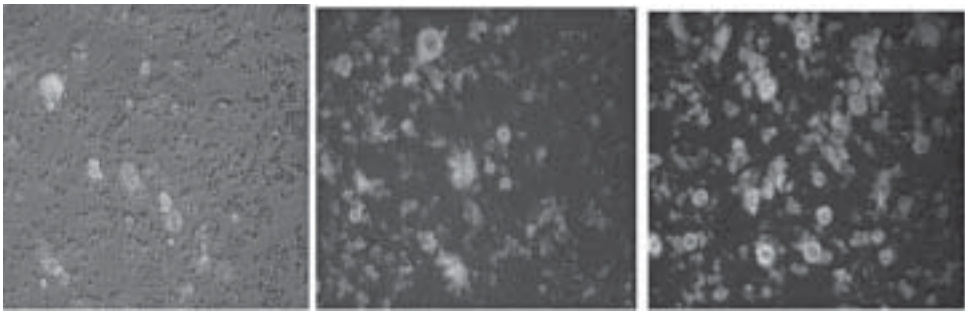
Survival and Replication of *S. aureus* Within Human Microvascular Endothelial Cells (HMECs)

To determine if *S. aureus* will replicate after being internalized by HMECs, the cells were washed after 1 hr of exposure to bacteria, and subsequently extracellular bacteria were killed with gentamicin sulfate solution (100 μg/mL). One hour after gentamicin exposure was considered the zero time point, and the number of surviving intracellular bacteria was determined after 1, 3, 5, and 24 hr. In the quantitative study, the number of bacteria engulfed by HMECs increased with increasing bacterial concentration. This is shown by the concentration values (number of colony forming units per milliliter, cfu/mL) at time zero in Fig. 3a. The number of internalized bacteria also



a. (-) Cytochalasin D b. (+) Cytochalasin D

FIGURE 1 **Uptake of *S. aureus* 3 hr After Exposure to HMECs. (a) In the Absence of Cytochalasin D and (b) After Exposure to Cytochalasin D.**



a. b. c.

FIGURE 2 **Uptake of Microspheres After (a) 1 hr of Exposure to HMECs. (b) 3 hr of Exposure to HMECs. (c) 5 hr of Exposure to HMECs.**

increased with time even after HMECs were exposed to cytochalasin D (Fig. 3a,b). The effect of cytochalasin D on the phagocytic ability of HMECs was very profound–approximately 20 times more bacteria were engulfed by HMECs which were not treated with cytochalasin D than those treated (Fig. 3c).

Effect of VCN Microspheres or Solution on Internalized *S. aureus* (Prophylactic Treatment)

As observed in the bacterial uptake and qualitative study, 1-hr exposure was enough for the endothelial cells to engulf a considerable amount of particulate matter. Uptake of bacteria or microspheres continued until saturation between 3 and 5 hr of exposure. Uptake of drug-loaded microspheres within the first hour would, therefore, not interfere with uptake of bacteria by the endothelial cells. The rate of intracellular growth of *S. aureus* was diminished by the presence of drug solution or microspheres. Using the intracellular bacterial concentration in the absence of drug treatment as a reference level, the prophylactic treatment of *S. aureus* infected HMECs showed that encapsulated VCN decreased the intracellular bacterial load by 73%, while the solution form decreased the bacterial load by 32% after 5 hr of incubation (SA: HMECs/100:1). There was a significant decrease in intracellular bacterial load at all time points by encapsulated VCN. ($p < 0.05$, Fig. 4a)

Immediate Treatment

Treatment of endothelial cells immediately after infection prevents excessive intracellular bacterial multiplication before the drug takes effect. A similar situation as in the prophylactic treatment was observed when HMECs were treated with drug solution or microspheres immediately after bacterial uptake. In the

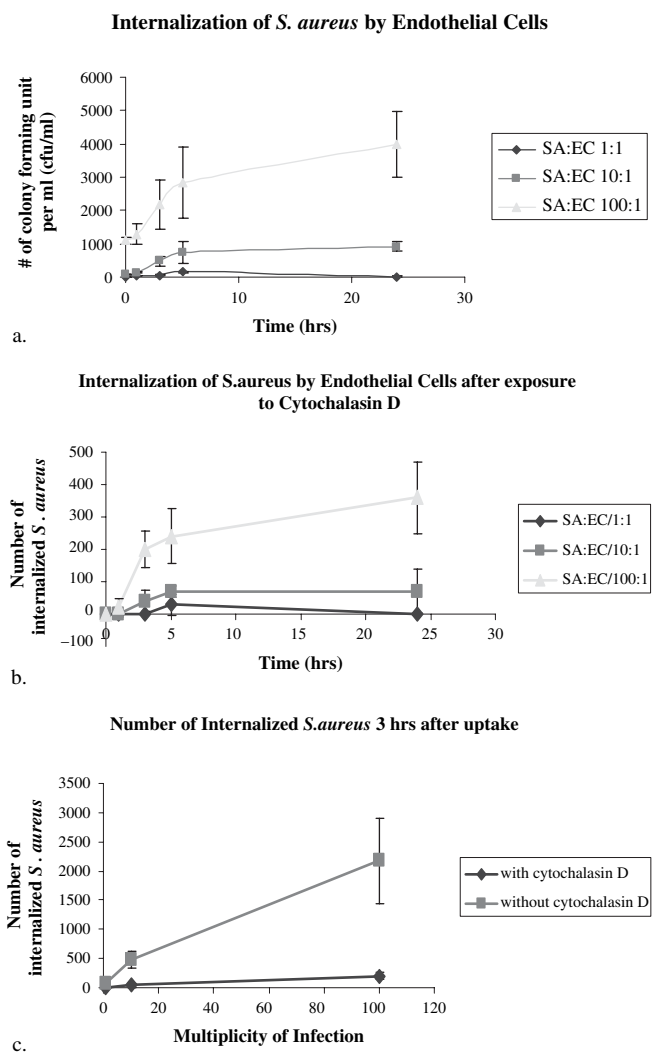


FIGURE 3 **Internalization and Survival of *S. aureus* (SA) Within Endothelial Cells (EC) (a) In the Absence of Cytochalasin D (b) After Cytochalasin D Treatment (c) Comparison of Uptake of *S. aureus* by Cytochalasin D Treated HMECs to Untreated Cells. Mean ± SD (n = 3).**

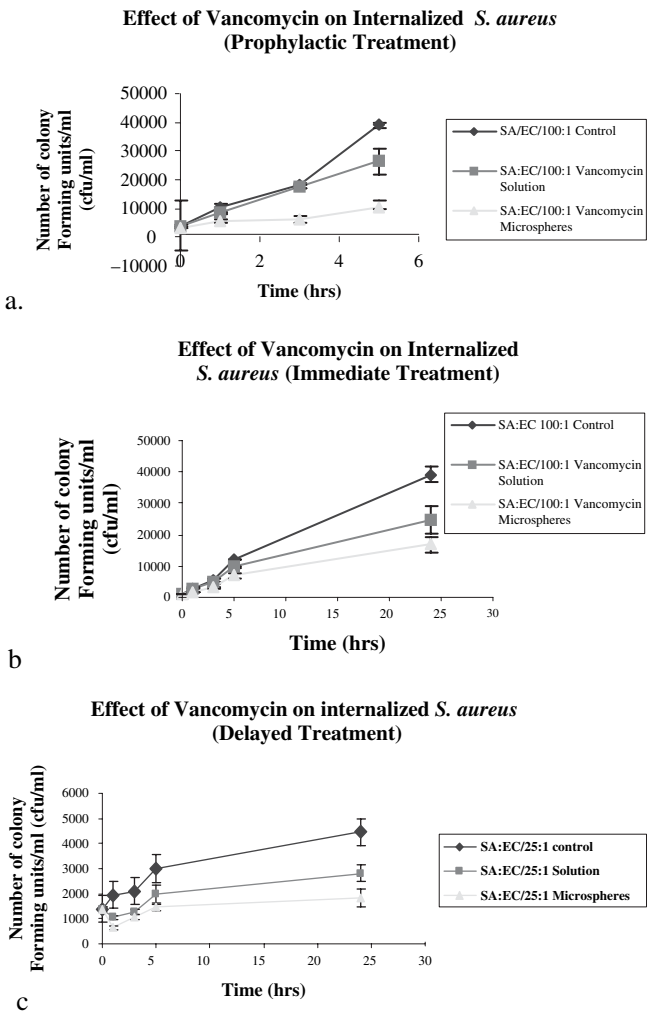


FIGURE 4 **Effect of VCN Microspheres or Solution on Intracellular *S. aureus* (SA) After (a) prophylactic treatment (b) Immediate Treatment (c) Delayed Treatment. Mean ± SD (n = 3).**

presence of drug solution or microspheres intracellular bacteria still multiplied, however, to a lesser extent than the controls. HMECs treated immediately after *S. aureus* uptake, showed a 57% decrease in bacterial load by VCN microspheres compared to a 37% decrease by VCN solution after 24 hr of incubation. The microsphere formulation was more effective in reducing the intracellular bacterial load than the solution form. However, a significant difference between the two was seen only at 5 and 24 hr. ($p < 0.05$, Fig. 4b).

Delayed Treatment

In delaying the treatment of *S. aureus* infected HMECs, it was observed that there was a slight decrease in bacterial load during the first hour of treatment. The cells, which did not receive any drug treatment, did not show this initial decrease indicating a drug effect. The intracellular bacterial load, however, continued to increase with time after the first hour. Both drug solution and micospheres decreased the intracellular bacterial load. Only the VCN microspheres were able to significantly decrease the bacterial load, and this occurred only at 5 and 24 hr posttreatment ($p < 0.05$). Figure 4c summarizes these results.

CONCLUSION

It can be concluded from the above results that endothelial cells, though not professional phagocytes, do internalize particulate matter appreciably. In these experiments endothelial cells were able to internalize both drug-loaded microspheres and bacteria in a time and concentration-dependent manner. Bacterial uptake was, however, impeded by the presence of cytochalasin D, confirming that the mechanism of uptake by endothelial cells is by phagocytosis through actin polymerization. Having established that endothelial cells do take up bacteria and microspheres separately, the next step was to allow the cells to engulf both bacteria and drug-loaded microspheres in vitro, and to evaluate the effect of the drug on intracellular bacteria. This group of experiments has shown that endothelial cells do internalize bacteria, drug solution, and microspheres. It was also shown that, irrespective of the treatment, the drug in the microsphere formulation was more effective than the solution in killing intracellular bacteria. The next appropriate step would be to evaluate a similar situation in vivo. Before in vivo experiments are done, however, it would be necessary to determine if *S. aureus*, when internalized, is exocytosed. It would also be interesting to know if bacterial cell wall components do affect endothelial cell monolayer integrity.

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