Immunopharmacology and Immunotoxicology, 28:601–607, 2006

ISSN: 0892-3973 print / 1532-2513 online
DOI: 10.1080/08923970601067045

informa
healthcare

A Pilot Study of an Anti-MRSA Bio-Engineered Lacteal Complex (Anti-MRSA BLC) in a Murine Septicemia Model

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Methicillin-resistant *Staphylococcus aureus* (MRSA) is an important pathogen of humans and other animals, causing septicemia, abcessation, toxemia, and other infectious diseases. Refined bioengineered lacteal complex (BLC), made specifically against MRSA, is a novel complex of low molecular weight immunogenic and antimicrobial molecules. It was evaluated *in vivo* using a mouse model of MRSA-induced peritonitis. Intraperitoneal dosing of anti-MRSA BLC demonstrated a therapeutic effect (83% survival) against an intraperitoneal MRSA challenge that caused 100% mortality in untreated animals. Anti-MRSA BLC is a promising therapeutic modality for MRSA infection.

Keywords Bio-engineered Lacteal Complex (BLC), Methicillin-Resistant *Staphylococcus Aureus (MRSA)*, Murine Peritonitis, Sepsis, Toxemia.

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (*MRSA*) is an increasingly common cause of life- and limb-threatening infections throughout the world.[1–3] It is now one of the most common causes of nosocomial and community-acquired infections and has developed a stable resistance to many antibiotics.[4] Since 1961, when *MRSA* was first reported as a clinical concern, it has become a

Dr. Stoff is the lead inventor of BLC and does not have any financial interest in Berlett, Inc. Berlett, Inc. does not have any financial interest in BLC. Dr. DeYoung and Dr. Nix have nothing to declare.

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serious problem with increasing numbers of cases and associated morbidity and mortality.[5,6]

A filtrate of colostrum and whey from antigen infused dairy cows has been used experimentally since the late 1950s in humans and animals.[7] Empirical and anecdotal evidence suggests enhanced immune-boosting properties compared with typical colostrum and whey products. From the beginning, this material appeared to restore immune function in people and animals suffering from conditions related to immune dysfunction including those suffering from acute, chronic, and degenerative diseases. Further research and development has yielded a novel (U.S. Patent Application No. 11/122,231) bioengineered lacteal complex (BLC) that utilizes new technology for its induction, purification, and characterization of molecular components. Anti-*MRSA* BLC contains specific, low molecular weight molecules that have antimicrobial properties against *MRSA* and also enhances immune function against *MRSA* infection.

The present study was undertaken to investigate *in vivo* effects of anti-*MRSA* BLC using an intraperitoneal systemic sepsis model.

MATERIALS AND METHODS

Bacterial Strain

A clinical isolate of a community acquired *MRSA* was utilized. Susceptibility testing using the Vitek® instrument at a certified clinical laboratory against 17 antibacterial agents showed susceptibility to gentamicin, nitrofurantoin, rifampin, vancomycin, tetracycline, and trimethoprim/ sulfamethoxazole.

Bacterial suspensions of *MRSA* (in log growth phase) in 3% Todd Hewitt media (BD, BBL ref # 211736, Sparks, MD, USA) were freshly prepared from an overnight culture on 5% sheep blood agar (Remel Blood Agar, ref 01202, Lenexa, KS, USA). The optical density was adjusted to 8% transmittance using a Vitek® colorimeter and this correlated with a 1×10^{10} per mL bacterial count. The inoculum concentration was confirmed by quantitative serial dilution with the spread plate method.

Anti-*MRSA* BLC

The anti-*MRSA* BLC material used in the study was specifically manufactured for that purpose making use of a new (patent applied for) technology. Chemically free raised cows went through immune induction using *MRSA* cell fragments along with cytokine adjuvants, inoculated into the wall of the udder with a CO_2 powered needleless "gun." After gathering milk from the cows, the milk was processed to remove all material with a molecular weight greater than 100 kD. The 100 kD filtrate was then evaluated for antimicrobial effect against *MRSA*, with growth inhibition curves on SRBC plates, as one

bio-logical marker for immune induction. After purifying the material it was standardized to 8 mg/mL of solids and assayed for its content of key molecules including defensins, granulysins, and specific transfer factors.

Animals

Young (10 week old, 24+/–2 g) BALB/c mice were used in this study. The study was approved by the University of Arizona Institutional Animal Care and Use Committee and the Institutional Bio-Safety Committee. Animal experiments were conducted at the University of Arizona Animal Care Facility and procedures adhered to all applicable standards for animal care. The mice were kept 3 to a cage and all the animals had unrestricted access to food and water throughout the study. They were kept in a BL 3 facility at standard temperatures and humidity. Each *MRSA*-dose test group consisted of 3 male and 3 female mice in order to establish the concentration of *MRSA* needed to induce terminal morbidity. The anti-*MRSA* BLC therapeutic group consisted of 12 male and 12 female mice. Animals demonstrating terminal morbidity (only minimally responsive to stimuli, labored breathing, and severe dehydration) were euthanized. Since BLC is a known immunogen, which is an important part of its mechanism of action, mice with normal immune systems were used.

In Vitro Experiments

Electrophoresis was used to evaluate the presence of peptides and molecules below 100 kD in the study drug materials. The presence of specific small molecular peptides is known to modulate the immune system. Over 100 different molecules were found to be present which constituted the 12 major bands demonstrated in the 18 % tris-glycine electrophoresis gel. Many of the molecules were identified by mass spectroscopy, amino acid sequencing, and other technologies. These molecules included cytokines, mini-cytokines, defensins, granulysins, transfer factors, antibody fractions, and lactoferrin.

Flow cytometry experiments were done on whole blood to calculate a theoretical maximal safe dose of the BLC based upon its immunogenic effects. For these experiments increasing amounts of BLC were added to 100 μL aliquots of whole blood, and at hourly intervals the blood was analyzed by flow cytometry for lymphocyte, monocyte, and neutrophil cell activation by measuring CD69 levels and cell movement. The activation curves were plotted and the optimal dose was calculated. Once the concentration of BLC exceeded 10% of the total volume there was less activation and an increase in cellular apoptosis was noted.

The dose utilized for the mouse model was determined to be 10% of the estimated blood volume (0.13 mL). Groups of 3 male and 3 female mice were

administered intraperitoneal doses of 0.13 mL anti-*MRSA* BLC for acute assessment. This group was closely observed for 2 weeks for their level of activity, responsiveness, feeding, and changes in body weight and showed no deviation from the expected normal behaviors.

Mouse Peritonitis Sepsis Model

The model chosen for this study was that of intraperitoneal systemic sepsis. This model has been well described and is a standard method of testing antimicrobial agents.[8,9] The lower abdomens of the mice were swabbed with alcohol and briefly allowed to dry. The mice were then inoculated in their left lower quadrant, intraperitoneally with of 0.1 mL of *MRSA* inoculum. Preliminary studies demonstrated that a 1×10^{10} cfu/mL suspension of *MRSA* (infective dose of 1×10^9 bacteria) induced 100% mortality, and this was used for the control and therapeutic groups included in this study. The control group, which received intraperitoneal *MRSA*, was observed every 2 hr and their apparent health recorded. The test group received the same inoculum of *MRSA* but they also received anti-*MRSA* BLC.

The test group was given an intraperitoneal injection of 0.13 mL anti-*MRSA* BLC, immediately after the bacterial challenge. Based upon *in vitro* studies, the effect of the BLC wanes by 4 hr. For mice in the test group that became significantly incapacitated, further doses of anti-*MRSA* BLC were given at 4-hr intervals. The mice were given no more than three doses of anti-*MRSA* BLC per day. At the end of the study the mice were humanely euthanized.

Measurement of Impairment

The mice were observed three times per day and their apparent health was recorded. The endpoint of the study was either death or "severely moribund" at which point the mice were sacrificed. However, data on their relative levels of impairment were recorded as part of the observational report. "Impaired" mice were defined as having diarrhea and mild dehydration. They still reacted normally to stimuli and continued to eat and drink normally. These mice could receive further doses of anti-*MRSA* BLC up to three doses per day. Moribund mice had severe diarrhea and/or severe dehydration. They reacted sluggishly or not at all to stimuli, had labored breathing, and did not try to eat or drink.

Statistical Analysis

Statistical analysis was accomplished using SAS version 8.2 (SAS Inc., Cary, NC, USA). Fisher's exact test was used to compare frequency data.

RESULTS

Figure 1 demonstrates the rapid induction of terminal morbidity that occurred in the untreated mice following intraperitoneal inoculation with the *MRSA*. They developed severe diarrhea very quickly followed by dehydration. They then became lethargic, obtunded, and all 6 died within 12 hr. Several groups of mice were required to establish the dose of *MRSA* needed. An intraperitoneal injection of 0.1 cc of 1×10^{10} cfu/mL caused 100% terminal morbidity.

Among anti-*MRSA* BLC treated mice, 20/24 (83%) survived compared with 0/6 (0%) of untreated mice ($p < 0.001$). After an initial 48 hr, post-*MRSA* inoculation, none of the surviving mice showed any lingering signs of illness or dehydration. They were observed to feed and drink normally, respond normally to stimuli, and their skin turgor returned to normal. After the 8:00 a.m. observation on the 8th day, mice were all humanely euthanized.

All female mice survived compared with 8/12 (67%) of male mice. Given the small sample size this difference in survival by sex was not statistically significant ($p=0.093$) Table 1 shows the morbidity response of the male mice given an inoculum of *MRSA* and then treated with anti-*MRSA* BLC. Two male mice were more significantly impaired and required reinjection with the anti-*MRSA* BLC of which 1 survived. The survival rate of the female mice was 100% (12 of 12 mice). One female mouse was more significantly impaired and required reinjection with the anti-*MRSA* BLC followed by a full recovery.

DISCUSSION

MRSA represents a common cause of nosocomial infections and in the past decade has emerged as an important cause of community acquired infections. The frequency of methicillin-resistance among isolates of *S. aureus*

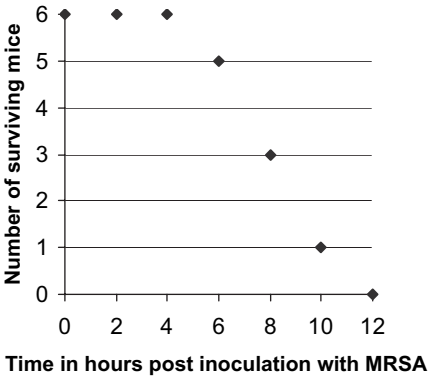

Figure 1: Number of surviving control (nontreated) mice postinoculation with MRSA.

Table 1: Morbidity response of the male mice given an inoculum of MRSA and then treated with anti-MRSA BLC.

Study day	Time	Normal	Impaired	Moribund	Deceased	BLC reinjection
Day 1	8:00 am	12				
	noon		12			
	4:00 pm		10	1	1	2
Day 2	8:00 am	8	1		2	1
	noon	7	2			2
	4:00 pm	7	2			2
Day 3	8:00 am	8			1	
	noon	8				
	4:00 pm	8				

All surviving mice (n = 8) were considered "normal" on days 4–8.

approximates 50% of hospital isolates and 15% of community acquired isolates. *MRSA* infections have been associated with greater health care expenditures and increased mortality compared with methicillin-susceptible *S. aureus*. Although vancomycin is considered the treatment of choice for serious infections involving *MRSA*, issues relating to therapeutic failures, poor penetration in certain body sites, and vancomycin resistance are major concerns. Recent introductions of the oxazolidinone, linezolid, and the lipopeptide, daptomycin, have provided new options; however, the role of these agents is often debated. Many problems with therapeutic management of *MRSA* infections remain.

BLC represents a new therapeutic direction by inducing an immunological response against the microbe for which it was made. An immune counter-attack by the host may limit the impact of resistance and may prove to be an important addition to the therapeutic armamentarium in the future.

In this study, females tended to respond better to anti-*MRSA* BLC therapy than male mice. Males appear more susceptible to *MRSA* infection, perhaps suggesting hormonal involvement in host response.[10] Sex hormones may influence bacterial growth and survival directly.[11] Estrogen is known to accelerate inflammation and the immune response in both mice and humans.[12] It has been postulated that estrogen has a differential effect on T and B cell mediated immune responses. Although survival differences by sex were not statistically significant ($p = 0.093$) in this study, future studies need to address this potential sex difference in response. If sex differences in response are confirmed, future studies will be required to ascertain whether a different dose or administration schedule of anti-*MRSA* BLC will overcome the difference.

ACKNOWLEDGMENT

This investigation was supported, in part, by a research grant from Berlett, Inc.

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