Improvement in Survival With Peptidyl Membrane Interactive Molecule D4B Treatment After Burn Wound Infection

Richard L. Gamelli, MD; Li-Ke He, MD; Hong Liu, MD; John D. Ricken, BS

Objective: To examine the effects of peptidyl membrane interactive molecule D4B in a murine model of lethal burn wound infection.

Experimental Design: Four experiments were performed: (1) growth inhibition assays of Pseudomonas aeruginosa treated with D4B, 0 to 100 µmol/L; (2) in vitro coculture of bone marrow cells with D4B, 0 to 100 µmol/L; (3) D4B treatment survival studies after burn injury only or burn wound infection in mice; and (4) peripheral white blood cell count, burn wound tissue bacterial culture, and burn wound morphological analysis at days 1, 2, and 3 after injury.

Setting: University medical center laboratory.

Subjects: Groups of B6D2F1 male mice (20 each) were studied.

Interventions: Full-thickness scald burn, 15% of total body surface area, with P aeruginosa topical infection, and subeschar injections of D4B at 200 µg or 0.25 mL of placebo per mouse at 2 and 24 hours after injury.

Main Outcome Measures: Animal survival after thermal burn wound bacterial infection, circulating leukocyte numbers, in vitro clonal cell culture of granulocyte-macrophage progenitor cells, and wound histopathological analysis.

Results: The survival rate in the D4B-treated group was nearly 2-fold greater than that in controls (P < .01) during 14 days of study. Bacterial quantitative wound cultures disclosed significant reductions in bacterial numbers at days 1, 2, and 3 in D4B-treated animals as compared with controls (P < .05 to < .01). D4B induced a dose-dependent inhibition of bacterial cell growth when added to in vitro P aeruginosa cultures (P < .01). Granulocyte-macrophage progenitor cell growth in culture was not altered by D4B treatment. D4B-treated animals displayed no signs of toxic effects or impairment in wound healing.

Conclusions: The peptidyl membrane interactive molecule D4B had the ability to improve survival after gram-negative burn wound sepsis via direct antimicrobial effects. Peptidyl membrane interactive molecules may offer the potential of alternative treatments to standard topical agents or in patients with drug-resistant microbes.

Arch Surg. 1998;133:715-720

Septic complications and the emergence of antimicrobial drug-resistant microbes represent serious risks to the burn and trauma patient. In 1985, 3 small antibiotic peptides were extracted from normal human neutrophils and named defensins. To date, more than 20 defensins have been identified in human and animal neutrophils, macrophages, or small intestinal (Paneth) cells. Defensins are peptides with 29 to 35 amino acid residues, including 6 invariant cysteines that form 3 intramolecular disulfide bonds and are present in high concentration in the azurophilic granules and phagocytic vacuoles of phagocytes. Their unusually broad antimicrobial spectrum encompasses gram-positive and gram-negative bacteria, many fungi, mycobacteria, spirochetes, and several enveloped viruses.

The antimicrobial properties of defensins result from their insertion into target cell membranes with the formation of voltage-sensitive channels. These antimicrobial peptides mediate oxygen-independent cytotoxic effects, 1 of the 2 general mechanisms of neutrophil killing. Defensins also may directly affect the host response. Reports have noted that defensins were able to blunt infection-mediated release of glucocorticoids via interactions with the corticotropin receptor. Defensins can be chemotactic for monocytes and modulate neutrophil...
MATERIALS AND METHODS

ANIMALS

Adult male B,D,F,1 mice, weighing 23 to 26 g, were purchased (Jackson Laboratory, Bar Harbor, Me) and were housed in a central animal research facility that maintains an environment of controlled temperature and relative humidity and a 12-hour light-dark cycle. Mice were cared for in cages that contained 4 mice each. Food and water were provided ad libitum. All procedures and the care and handling of our animals were reviewed and approved by the Institutional Animal Care and Use Committee. A total of 90 mice were used.

PEPTIDYL MEMBRANE INTERACTIVE MOLECULE D4B

The D4B (Demeter BioTechnologies Ltd, Research Triangle Park, NC) was prepared in sterile distilled water at concentrations of 0 to 100 µmol/L for the in vitro experiments, or 200 µg/0.2 mL for the in vivo treatments.

GRANULOCYTE-MACROPHAGE COLONY-FORMING CELL CULTURE

Granulocyte-macrophage colony-forming cell (GM-CFC) cultures with 25,000 marrow cells pretreated with D4B or control were performed in 35-mm plastic Petri dishes (Falcon, Lincoln Park, NJ). Cultures were supplemented with 25 µL of pooled mouse serum, obtained from postendotoxin mice, as a source of supermaximal colony stimulating. The cultures were performed in 1 mL of McCoy culture medium with 15% fetal bovine serum and 0.15% agar (Difco Laboratories, Detroit, Mich). Cultures were performed for 7 days at 37°C in a humidified 10% carbon dioxide and air atmosphere. Colonies were counted by means of a dissecting microscope at ×20, and aggregates containing 50 or more cells were scored as colonies. Cultures with D4B treatment were analyzed as a percentage of control cultures.

RESULTS

After randomization into groups of equal weight, animals were anesthetized with intraperitoneal administration of pentobarbital sodium (0.8 mg/10 g of body weight). The hair was clipped from the dorsum and a 15% total body surface area full-thickness burn was created by immersion of the dorsal skin in a 97°C to 100°C water bath for 7 seconds. Animals were then given an intraperitoneal injection of 2 mL of isotonic sodium chloride solution for resuscitation. A volume of 0.25 mL containing 1000 colony-forming units of Pseudomonas aeruginosa (American Type Culture Collection No. 19960) was directly and immediately applied on the scalded area. The P aeruginosa was grown in tryptic soy broth incubated for 18 hours at 37°C in a shaking water bath, spun, and washed with phosphate-buffered saline twice in a refrigerated centrifuge and diluted to appropriate concentrations. Bacterial concentrations were estimated spectrophotometrically and then verified by placing serial dilutions on tryptic soy agar plates and performing quantitative bacterial culture.

WHITE BLOOD CELL COUNT AND DIFFERENTIAL COUNT

Peripheral blood for leukocyte number determination and differential counts was obtained via tail vein bleeding. Total white blood cell counts were performed by counting in a hemocytometer after lysis of erythrocytes with 0.1N hydrochloric acid. Differential counts were carried out on 100 leukocytes obtained from a blood smear stained with a modified hematoxylin-eosin technique (Diff-Quik Solution, Baxter Inc, Miami, Fla). The absolute number of neutrophils, eosinophils, monocytes, or lymphocytes was calculated by comparing the percentages obtained on differential count with the total leukocyte number.

BURN WOUND BACTERIAL QUANTITATIVE CULTURE

Wound tissues were harvested aseptically in duplicate immediately after the animals were killed. Specimens were analyzed as a percentage of control cultures.

adhesion, superoxide anion generation, and phagocytosis. Additionally, defensins have been reported to increase histamine release from mast cells and to stimulate DNA synthesis and growth in epithelial cells as well as fibroblasts without apparent cytotoxic effects. Moreover, defensins have been reported to, alone or in combination with other proteins, inhibit the endotoxin effects of lipopolysaccharides or whole bacteria, which included blocking endotoxin priming of neutrophils for enhanced arachidonate release.

Clinically, increased levels of defensins have been found in the plasma of septic patients. It has been suggested that the plasma levels of human neutrophil defensins 1 to 3 may serve as specific markers of neutrophil-mediated inflammation. In the series of studies we report herein, a specific peptidyl membrane interactive molecule, DB4, synthesized 14 to 37 amino acid compounds based on the naturally occurring defensin molecules and was able to significantly improve survival after burn wound infection with Pseudomonas aeruginosa. This improved survival with D4B administration was not associated with any apparent adverse responses in treated animals. In vitro D4B demonstrated dose-dependent inhibition of P aeruginosa growth while showing no such inhibition of bone marrow progenitor cell growth. With the need to limit drug-related toxic effects in critically ill burn and trauma patients and the continued emergence of drug-resistant microbes, such compounds as these peptidyl membrane interactive molecules may be efficacious in the clinical setting.

Burned animals that received peptidyl membrane interactive molecule D4B treatments showed no general signs of toxic effects and maintained normal feeding and activity patterns and body weights. Animals with burn wound infection receiving D4B treatments did not manifest any additional findings beyond...
that of their infection. The local injection of D4B was not associated with any alteration of the wounds in burn-only animals or in animals with burn wound infections.

The in vitro studies examining the antibacterial abilities of the D4B compound used in these experiments revealed a dose-dependent inhibition of \textit{P. aeruginosa} growth. At the 2 highest concentrations tested, we found a complete suppression of bacterial growth. At the 2 highest concentrations tested, alterations in in vitro GM-CFC as an index of potential host tissue toxic effects. Bone marrow cells, obtained from normal mice, were cultured in D4B at concentrations of 0, 0.5, 1, 5, 10, 50, and 100 µmol/L. Alterations in vitro GM-CFC clonal growth as a result of D4B were determined after 7 days of culture.

Survival Studies

Survival studies were carried out in groups of 20 animals each receiving burn wound infection treated with D4B (B + I + D) or vehicle control (B + I). Animals were administered subeschar injections of D4B, 200 µg per animal, or vehicle control at 2 and 24 hours after injury. A third group, consisting of 10 burned-only animals, was treated with D4B, 200 µg per animal (B + D). All groups were followed up twice daily to day 14 after injury for survival. Using this model, we previously observed that animals surviving to day 14 were long-term survivors.

Burn Wound Analysis

In a separate series of experiments, burned and infected animals were treated with D4B (B + I + D) or vehicle control (B + I) as in experiment 2. Groups of 6 animals each were then sampled at days 1, 2, and 3 after burn infection and treatment. Burn wound tissue bacterial culture and burn wound morphological analysis were performed. Day 1 animals were analyzed 2 hours after the final D4B injection. Peripheral white blood cell counts and differential cell counts were also performed in these groups of animals.

Figure 1

\textbf{Burn Wound Histological Examination}

Additional wound samples were taken as described above and used for paraffin slides with hematoxylin-eosin and Gram staining. Analysis was performed by histopathological scoring of wound specimens by 2 independent pathologists (L.-K.H. and H.L.). Evaluation was based on the depth of “invasion” and the cellular response. The microbial status of the wound was classified on the basis of the density and depth of penetration of microorganisms according to the staging system of Pruitt and Goodwin, where stage I equals colonization; Ia, superficial colonization; Ib, penetration; Ic, proliferation; II, invasion; Ila, microinvasion; IIb, generalized invasion; and IIc, microvascular invasion. The inflammatory cellular response was graded as 1, few; 2, some; 3, many; and 4, excessive numbers of inflammatory cells being present with the wound.

\textbf{Survival Studies}

The total leukocyte counts in B + I animals were significantly higher than in B + I + D animals on day 1 (B + I: 13.1 ± 0.9 vs B + I + D: 9.1 ± 0.6 × 10^9/L; \textit{P}<.01). However, there was a reversal in this finding by day 2 (B + I: 7.6 ± 1.0 vs B + I + D: 11.5 ± 0.8 × 10^9/L; \textit{P}<.05). On day 3, leukocyte numbers were nonsignificantly different in B + I animals compared with infected burned animals receiving D4B (Table). Absolute neutrophil counts, eosinophil values, and monocyte numbers did not show a consistent response pattern for animals receiving D4B. The changes in lymphocyte numbers paralleled the response for total leukocyte counts. On day 1, lymphocyte values in B + I animals were significantly greater than those in B + I + D animals (B + I: 8.0 ± 0.8 vs B + I + D: 5.2 ± 0.1 × 10^9/L; \textit{P}<.01), and at day 2 there was a reversal in the relative differences (B + I: 3.8 ± 1.1 vs B + I + D: 8.6 ± 0.7 × 10^9/L; \textit{P}<.05).
The subeschar injection of D4B did substantially alter the number of *P. aeruginosa* organisms recovered on quantitative wound culture. The numbers of bacterial colony-forming units obtained on wound biopsy (\(3 \times 10^6/g\)) were significantly less in B+I+D animals than in B+I animals at all time points analyzed (day 1, B+I: 0.9 ± 0.3 vs B+I+D: 0.03 ± 0.01 \(10^6/g\); \(P<.01\); day 2, B+I: 199 ± 72 vs B+I+D: 2.3 ± 0.9 \(10^6/g\); \(P<.05\); day 3, B+I: 158 ± 65 vs B+I+D: 3.1 ± 1.2 \(10^6/g\); \(P<.05\)) (Figure 3). Interestingly, we could not determine a consistently observable trend by morphological criteria related to D4B therapy as compared with nontreated burned infected animals for stage of wound infections and the histological presence of bacteria (data not shown). Infiltrating cell type at the viable tissue interface adjacent to the eschar was predominantly neutrophils in both groups of animals receiving burn wound infections. There was no observable pattern of infiltrate that was different among D4B-treated vs nontreated animals.

**Comment**

Granulocytes are critical effector cells in host defenses against microbial infection. They destroy invading microorganisms by 2 principal means—oxygen-dependent and oxygen-independent mechanisms. The latter are mediated by antimicrobial peptides and proteins stored within the cytoplasmic granules of neutrophils. The defensin family of peptides are membrane interactive molecules that contribute to oxygen-independent antimicrobial mechanism. The properties of their broad antimicrobial spectrum and chemical resistance and lack of antigenicity suggest that they may have clinical utility. To date, studies have characterized the nature of these compounds, their purification, biology, biochemistry, and biosynthesis. Several studies have evaluated the production and levels of defensins during septic events. The significance of the data we report here is that the peptidyl membrane interactive molecule D4B, a synthetic membrane interactive antibiotic peptide molecule, has shown the ability in a model of burn wound infection to alter mortality and reduce the number of recoverable bacteria in the wound. This was not associated with any observable systemic or wound-related complications.

The naturally occurring membrane interactive molecules, defensins, are endogenous antibiotic peptides and make up about 5% to 7% of the total protein of human...
neutrophils and 30% to 50% of the protein in the azurophilic granules.4,31,32 It has been shown that most of the microbicidal peptide activity of neutrophils is present in azurophilic granules32-34 whose contents are predominantly released into phagosomes rather than extracellularly.35 The concentrations of defensins are as high as 1 to 10 mg/mL in phagocytic vacuoles containing ingested microbes.3 The plasma levels of defensins, however, are only about 40 ng/mL normally and rise to values of 1 µg/mL during severe infection.28,30 It has been demonstrated that at a neutrophil concentration of 2 × 10^6/mL, only 5% of neutrophil defensin is released extracellularly, which yields a defensin concentration of about 6 µg/mL, a concentration below that required for the killing of Escherichia coli in vitro.1,31 The intriguing thought with peptidyl membrane interactive molecule D4B treatment is that exogenous antimicrobial peptides could be delivered at concentrations that are microbicidal at sites of infection and that they would far exceed concentrations of endogenously released peptides, not be associated with the release of necrotic factors, and be useful in states of neutrophil dysfunction.

Naturally occurring antibiotic peptides are not stable and do not retain their activity in the extracellular milieu as they are inactivated by physiological concentration of sodium chloride and divalent cations.36,37 Our observations demonstrated that the peptidyl membrane interactive molecule compound we used was bioactive and retained its antimicrobial capacity when injected at the wound site. The dose that we used was based on our in vitro studies of peptidyl membrane interactive molecule in the bacterial inhibition culture experiments and our desire to achieve similar levels with in vivo delivery. Additional doses beyond those we used might have provided further survival advantage than that which we achieved.

Defensins are abundant in human,1 rabbit,36 guinea pig,36 and rat granulocytes. Mouse neutrophils, however, lack appreciable amounts of defensins.38 Additionally, murine bone marrow cells have been reported to lack the messenger RNA encoding the defensins expressed by murine small-intestinal Paneth cells.92 These observations suggest that the effects of enhanced survival and reduced bacterial burden at the wound site were attributable to a direct effect of the peptidyl membrane interactive molecule that we used. Further, as we saw no consistent pattern of change in circulating leukocytes or wound cellular inflammatory recruitment, our results suggest that the response was primarily an antibacterial peptidyl membrane interactive molecule-mediated killing.

The capacity of peptidyl membrane interactive molecules to improve survival after lethal burn wound infection without apparent toxic effects in our studies was significant. The opportunity to develop alternatives to standard antimicrobial treatment with the development of microbial drug resistance is assuming ever-greater importance in clinical medicine. Additionally, we have not yet examined whether alternative uses might be of value, such as aerosolized delivery of peptidyl membrane interactive molecules in a setting of bacterial pneumonia. Further studies are required to delineate the potential of peptidyl membrane interactive molecules to support the care of critically ill individuals who develop infectious complications and the ability to improve patient outcome.

This study was supported by the Mary Anne Miller Burn Fund at Loyola University Medical Center, Maywood, Ill, and a grant from Demeter BioTechnologies Ltd, Research Triangle Park, NC.


Corresponding author: Richard L. Gamelli, MD, Burn and Shock Trauma Institute, Loyola University Medical Center, 2160 S First Ave, Maywood, IL 60153 (e-mail: rgamell@wpo.it.luc.edu).

REFERENCES


13. Ogata K, Linzer BA, Zuberi RI, Ganz T, Lehrer RI, Catanzaro A. Activity of de
fensins from human neutrophilic granulocytes against Mycobacterium avium-
14. Lehrer RI, Daher K, Ganz T, Selsted ME. Direct inactivation of viruses by MCP-1
and MCP-2, natural peptide antibiotics from rabbit leukocytes. J Virol. 1985;54:
467-472.
15. Daher KA, Selsted ME, Lehrer RI. Direct inactivation of viruses by human granu-
16. Lehrer RI, Barton A, Ganz T. Concurrent assessment of inner and outer mem-
brane permeabilization and bacteriolysis in E. coli by multiple-wavelength spec-
17. Kagan BL, Selsted ME, Lehrer RI, Ganz T. Concurrent assessment of inner and outer mem-
brane permeabilization and bacteriolysis in E. coli by multiple-wavelength spec-
18. Fuji G, Selsted ME, Eisenberg D. Defensins promote fusion and lysis of nega-
19. Elsbach P, Weiss J. A reevaluation of the roles of the O2-dependent and O2-
independent microbicidal systems of phagocytes. Rev Infect Dis. 1983;5:843-
853.
20. Zhu QZ, Singh AV, Bateman A, Esch F, Solomon S. The corticostatic (anti-
ACTH) and cytotoxic activity of peptides isolated from fetal, adult and tumor-
21. Zhu QZ, Singh AV, Bateman A, Esch F, Solomon S. The corticostatic (anti-
ACTH) and cytotoxic activity of peptides isolated from fetal, adult and tumor-
22. Hu J, Bennett HP, Lazure C, Solomon S. Isolation and characterization of corti-
costatic peptides from guinea pig bone marrow. Biochem Biophys Res Com-
23. Territo MC, Ganz T, Selsted ME, Lehrer RI. Monocyte-chemotactic activity of de-
24. Yamashita T, Saito K. Purification, primary structure, and biological activity
25. Yamashita T, Saito K. Purification, primary structure, and biological activity
26. Murphy CJ, Foster BA, Mannis MJ, Selsted ME, Reid TW. Defensins are mito-
of granulocytes differ in interaction with endotxin: comparison of bactericidal/
5410.
28. Panyutich AV, Panyutich EA, Krapivin VA, Baturevich EA, Ganz T. Plasma defen-
sin concentrations are elevated in patients with septicemia or bacterial meningi-
29. Panyutich AV, Voitenok NN, Lehrer RI, Ganz T. An enzyme immunoassay for hu-
30. Shiomi K, Nakazato M, Ihi T, Kangawa K, Matsuo H, Matsukura S. Establish-
ment of radioimmunoassay for human neutrophil peptides and their increases
in plasma and neutrophil in infection. Biochem Biophys Res Commun. 1993;95:
1336-1344.
32. Ganz T. Extracellular release of antimicrobial defensins by human polymorpho-
33. Rice WG, Ganz T, Kinkade JM, Selsted ME, Lehrer RI, Parmly RT. Defensin-rich
34. Rest RF, Cooney MH, Spitznagel JK. Bactericidal activity of specific and azuro-
phil granules from human neutrophils: studies with outer-membrane mutants of
35. Leffell MS, Spitznagel JK. Intracellular and extracellular degradation of human
polymorphonuclear azurophil and specific granules induced by immune com-
37. Lehrer RI, Lichtenstein AK, Ganz T. Defensins: antimicrobial and cytotoxic pep-
38. Selsted ME, Szklarek D, Lehrer RI. Purification and antibacterial activity of anti-
39. Selsted ME, Harwig SSL. Purification, primary structure, and antimicrobial ac-
40. Selsted ME, Lehrer RI. Purification and antimicrobial properties of three de-
60:3446-3447.
42. Ouellette AJ, LuValt JC. A novel mouse gene family coding for cationic, cysteine-
1990;265:9831-9837.

Surgical Anatomy

The rhomboids and the serratus, though antagonistic in that they pull the scapula in opposite
directions, work together in holding the vertebal border of the scapula to the thoracic wall.

1. Grant JCB. A Method of Anatomy: Descriptive and Deductive. 5th ed.
Baltimore, Md: Williams & Wilkins; 1952:180.