

Bacterial Load Predicts Healing Rate in Neuropathic Diabetic Foot Ulcers

LING XU, MD^{1,2}
 SUSAN V. McLENNAN, PHD^{1,3}
 LISA LO, BSC¹
 ANAS NATFAJI, MD⁴

THYRA BOLTON^{3,4}
 YU LIU, MD¹
 STEPHEN M. TWIGG, MD^{1,3,4}
 DENNIS K. YUE, MD^{1,3,4}

Despite improved treatment, a significant number of diabetic foot ulcers do not heal and eventually lead to amputation (1–5). The important local factors determining the healing rate of ulcers are pressure at the site of the ulcer, adequacy of blood supply, and infection. It is generally accepted that for optimal healing, infection needs to be treated. However, the definition of infection is arbitrary, and, in many borderline cases, clinicians will be uncertain whether antibiotic therapy is indicated. Edmonds et al. (6) showed, in a study of clinically uninfected diabetic foot ulcers, that, even in the absence of overt infection, antibiotic therapy reduced hospitalization and amputation. Conceptually, a high bacterial load by itself can retard ulcer healing by causing a wound environment not conducive to healing. Important factors that may play a role in this regard include secretion of metalloproteinases and their tissue inhibitors from the bacteria, compounds that can cause local tissue destruction (7). Little is known, however, about the effect of bacterial load on the healing rate of neuropathic diabetic foot ulcers. In this study, instead of arbitrarily deciding clinically whether infection was present, a quantitative microbiological method was used to examine the relationship between bacterial load in the wounds of diabetic neuropathic ulcers and the subsequent ulcer healing rate.

RESEARCH DESIGN AND METHODS

Wound fluid was obtained from 32 patients (22 male and 10 female) with neuropathic ulcers at the plantar surface of the foot. Patients were referred to the High Risk Diabetic Foot Clinic at the Diabetes Centre of Royal Prince Alfred Hospital in Sydney, Australia. The mean \pm SD age of the cohort was 60.0 ± 9.0 years with a diabetes duration of 14.6 ± 10.1 years and an A1C of $7.9 \pm 1.4\%$ (normal $<6.0\%$). All patients had vibration perception threshold >50 V and ankle brachial index >0.9 ; thus, their ulcers would be considered to be substantially neuropathic in nature. Their ulcers were relatively superficial at grade 0 or 1 and stage A or B, according to the Texas Grading System (8). All patients underwent regular ulcer care, provided by the multidisciplinary High Risk Foot Service, which included a minimum of one visit per week for debridement, dressing, and other aspects of treatment. Antibiotics had been prescribed in 83% of individuals, but not all cases would be considered infected by conventional criteria. The protocol was approved by the Sydney South West Area Health Service Ethics Committee.

Wound fluid collection and quantitation of bacterial load

At the initial visit, wound exudates were removed by flushing with saline, and necrotic tissues were removed by local de-

bridement. Wound fluid was then absorbed onto a sterile 1-cm² piece of Whatman filter paper (Whatman International, Kent, U.K.) by placing the disc on the most exudative area of the ulcer. When the disc became fully saturated, it was removed from the ulcer base and placed into a tube containing 100 μ l sterile PBS before storage at 4°C for 1–2 h. The sample was mixed, and 10 μ l of the supernatant was then serially diluted (10^{-2} to 10^{-7}), streaked onto blood agar plates, and aerobically incubated for 24 h at 37°C. Bacterial load was quantified by counting the number of colony-forming units (CFUs) on each plate. The bacterial species were identified by standard microbiological techniques, including gram stain, microscopic examination, and, where appropriate, a coagulase test and assessment for methicillin-resistant *Staphylococcus aureus* status.

To verify the reproducibility of sampling in a wound by this method, six sequential wounds were examined, each with triplicate samples from the same wound. In each wound, the within-wound variability in the measured CFU was low at $6.8 \pm 2.5\%$. In each of the six samples, we tested residual bacteria on the filter paper used for sampling, after fluid containing the PBS was removed, by directly applying culture of the paper onto culture plates—which, in each case, showed <50 colonies ($<0.5 \times 2 \log$ CFU and $<1\%$ of the measured CFU in the wound fluid sample).

Measurement of ulcer area

At each visit to the clinic, the ulcer was debrided and the ulcer borders traced onto sterile transparent film using an acetate pen. The tracing was digitized by scanning and the area calculated using National Institutes of Health image software (9). The wound healing rate over the following 28 days was calculated as: daily change in wound area (%) = [(area at visit 1 – area at visit 4)/(area visit 1)/28] \times 100 (10).

Statistical analysis was performed using the Number Cruncher Statistical System. Log-transformed data were analyzed for CFUs, as the data were normally distributed. Significance was accepted at $P < 0.05$.

From the ¹Discipline of Medicine, University of Sydney, Sydney, New South Wales, Australia; the ²Department of Geriatrics, Qi Lu Hospital, Medical College, Shandong University, Jinan, China; the ³Department of Endocrinology, Royal Prince Alfred Hospital, Sydney, Australia; and the ⁴Diabetes Centre, Royal Prince Alfred Hospital, Sydney, New South Wales, Australia.

Address correspondence and reprint requests to Dr. Susan McLennan, Department of Endocrinology, Royal Prince Alfred Hospital, W407, Blackburn Building, D06, University of Sydney, Sydney, NSW, Australia.

Received for publication 4 July 2006 and accepted in revised form 1 November 2006.

Abbreviations: CFU, colony-forming unit.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

DOI: 10.2337/dc06-1383

© 2007 by the American Diabetes Association.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

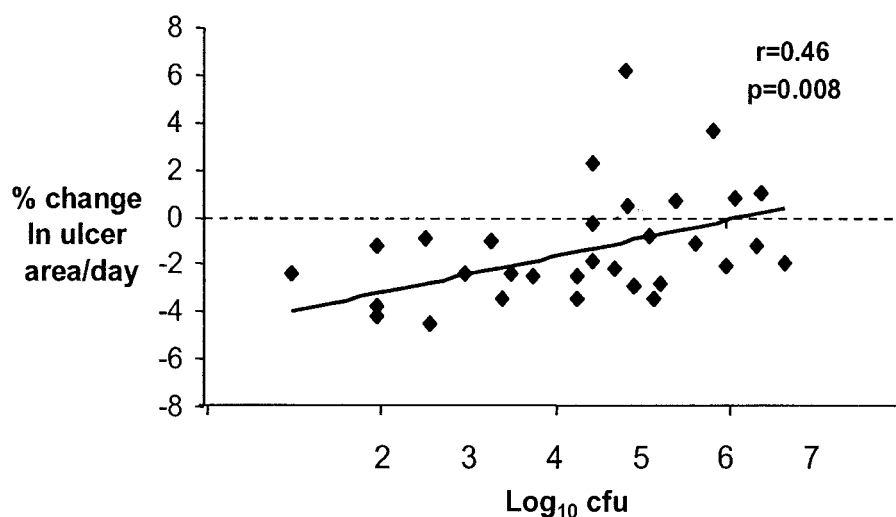


Figure 1—The relationship between initial viable bacterial load (CFU) and subsequent rate.

RESULTS— The mean initial area of the ulcers was 170.1 mm² (range 6.5–979.5). After 28 days of treatment, the ulcers had reduced in size by an average of 42% to 98.7 mm² (0–567.2). Wound healing rate showed a strong inverse relationship with logCFU ($r = 0.46$, $P = 0.008$) (Fig. 1). For each log order of CFU increase, ulcer healing was delayed by 44%. Poor wound healing, indicated by either no significant change or an increase in wound area over the 28 days, was observed in 13% of patients, and all of these had a CFU in the order of at least 10⁴ CFU.

The bacterial species present in the wound fluid were typical of those commonly found in diabetic foot ulcers. *S. aureus* and *Staphylococcus epidermidis* were present in 41 and 47% of the cases, respectively. None of the *S. aureus* isolates were methicillin-resistant *S. aureus* positive. Coliforms, streptococci, and *Bacillus* were also present in 29, 18, and 6%, respectively. The majority of ulcers (63%) contained more than one organism type, with the most common combination being *S. aureus* and *S. epidermidis*.

Glycemic control, as assessed by A1C level at presentation, was also negatively correlated with wound healing rate over the 28 days ($r = -0.41$, $P < 0.02$). However, there was no correlation between bacterial load and A1C (data not shown). On univariate analysis, neither patient age nor diabetes duration predicted wound healing rate ($P > 0.05$).

CONCLUSIONS— In this study, a quantitative measure of bacterial load is shown to be correlated with rate of dia-

betic foot ulcer healing. To our knowledge, this is the first time that such relationship has been described in diabetes. Previous research (11,12) in other disease states has indicated that reduced ulcer healing may occur as a result of the presence of certain types of bacteria (such as *S. aureus*), the presence of a complex mix of bacteria including anaerobes, or >10⁶ CFU/mg of tissue in neuroischemic ulcers. Our method of ulcer fluid sampling, from the reproducible CFU counts from the same wound, and others (13) have also shown that postdebridement wound fluid CFU highly correlates with CFU counts derived from tissue samples in the same wound.

Obviously, frank infection of diabetic foot ulcers with its associated tissue destruction is well-known to all who look after this group of patients. However, the positive correlation between bacterial load and healing lends support to the notion that high bacterial load may contribute to impaired wound healing. Being a cross-sectional study at only one time point in the natural history of diabetic foot ulceration, the primary culprit could not be determined, nor could the question of whether the observed relationship was a causal one. However, the continuous relationship between bacterial load and healing, observed without considering whether antibiotic therapy is used, may minimize the frequently encountered dilemma of whether to use antibiotic therapy in borderline clinical cases. Conceptually, there are many explanations as to how bacteria can impair wound healing. Increased activities of metalloproteinases and disturbed pattern of in-

flammatory cytokines and growth factors are some of the possibilities (7). On the other hand, the natural body defense system may be in a better position to eliminate bacteria in a healthy and healing wound. Insight into these possibilities can be obtained by further intervention studies using antibiotics or other therapies to modulate bacteria load, while the patterns of metalloproteinase activities, inflammatory cytokines, and growth factors are closely monitored. These are important studies that need to be performed in our quest to overcome the serious problem of foot ulceration in diabetes. Irrespective of the underlying mechanisms, our finding suggests that CFU count in wound fluid may be a useful adjunct investigation to identify patients who need more intensive therapy for their diabetic foot at an earlier stage.

Acknowledgments— We thank the staff of the Microbiology Department, the Diabetes Centre Podiatry staff (V.N., T.C., E.P., and D.W.) of RPA Hospital for their expert assistance, and the Diabetes Australia Research Trust for competitive grant support of the study.

References

1. Muller IS, De Grauw WJC, Van Gerwen WHEM, Bartelink ML, Van Den Hoogen HJM, Rutten GEHM: Foot ulceration and lower limb amputation in type 2 diabetic patients in Dutch primary health care. *Diabetes Care* 25:570–574, 2002
2. Boyko EJ, Ahroni JH, Smith DG, Davignon D: Increased mortality associated with diabetic foot ulcer. *Diabet Med* 13: 967–972, 1996
3. Moulik PK, Mtonga R, Gill GV: Amputation and mortality in new-onset diabetic foot ulcers stratified by etiology. *Diabetes Care* 26:491–494, 2003
4. Diouri A, Slaoui Z, Chadli A, El Ghomari H, Kebbou M, Marouan F, Farouqi A, Ababou MR: Incidence of factors favoring recurrent foot ulcers in diabetic patients. *Ann Endocrinol (Paris)* 63:491–496, 2002
5. Boulton AJ: The diabetic foot: from art to science: the 18th Camillo Golgi Lecture. *Diabetologia* 47:1343–1353, 2004
6. Edmonds M, Foster A: The use of antibiotics in the diabetic foot. *Am J Surg* 187: 25S–28S, 2004
7. Wang YY, Myhre AE, Pettersen SJ, Dahle MK, Foster SJ, Thiemermann C, Bjornland K, Aasen AO, Wang JE: Peptidoglycan of *Staphylococcus aureus* induces enhanced levels of matrix metalloproteinase-9 in human blood originating from neutrophils. *Shock* 24:214–218, 2005
8. Oyibo SO, Jude EB, Tarawneh I, Nguyen

- HC, Armstrong DG, Harkless LB, Boulton AJ: The effects of ulcer size and site, patient's age, sex and type and duration of diabetes on the outcome of diabetic foot ulcers. *Diabet Med* 18:133–138, 2001
9. National Institutes of Health Image: About NIH Image [online]. Available from <http://rsb.info.nih.gov/nih-image/Default.html>. Accessed February 2005
10. Sheehan P, Jones P, Caselli A, Giurini JM, Veves A: Percent change in wound area of diabetic foot ulcers over a 4-week period is a robust predictor of complete healing in a 12-week prospective trial. *Diabetes Care* 26:1879–1882, 2003
11. Bowler PG, Davies BJ: The microbiology of acute and chronic wounds. *Wounds* 11: 72–79, 1999
12. Bowler PG, Duerden BI, Armstrong DG: Wound microbiology and associated approaches to wound management. *Clin Microbiol Rev* 14:244–269, 2001
13. Gardner SE, Frantz RA, Saltzman CL, Hillis SL, Park H, Scherubel M: Diagnostic validity of three swab techniques for identifying chronic wound infection. *Wound Repair Regen* 14:548–557, 2006