Original Article

The Pathogenesis of *Staphylococcus aureus* Infection in the Diabetic NOD Mouse

Jeremy Rich and Jean C. Lee

Although *Staphylococcus aureus* is a major pathogen implicated in diabetic foot infections, little is known about the pathogenesis of this disease. A model of *S. aureus* infection in the hindpaw of nonobese diabetic (NOD) mice was developed. The experimental infection was exacerbated in diabetic mice (blood glucose levels ≥19 mmol/l) compared with nondiabetic mice, and the diabetic animals were unable to clear the infection over a 10-day period. Insulin-mediated control of glycemia in diabetic mice resulted in enhanced clearance of *S. aureus* from the infected tissue. Diabetic mice showed reduced tissue inflammation in response to bacterial inoculation compared with nondiabetic NOD animals, and this was consistent with the novel finding of significantly decreased tissue levels of the chemokines KC and MIP-2 in diabetic mice. Blood from nondiabetic and diabetic NOD mice killed *S. aureus* in vitro, whereas the bacteria multiplied in blood from diabetic mice with severe hyperglycemia. The impaired killing of *S. aureus* by diabetic mice was correlated with a diminished leukocytic respiratory burst in response to *S. aureus* in blood from diabetic animals. This animal model of hindpaw infection may be useful for the analysis of host defects in innate immunity that contribute to recalcitrant diabetic foot infections. *Diabetes* 54:2904–2910, 2005

Diabetes afflicts ~16 million individuals in the U.S., and it is estimated that 2 million of these individuals will eventually develop chronic foot ulcers and infections (1). The most common cause of hospitalization for diabetic patients is foot infections (1), and the susceptibility of the diabetic host to these infections is multifactorial. Diabetes results in numerous physiologic perturbations in the host such as neuropathy and vasculopathy, and these can play a prominent role in the development of the diabetic foot (2).

Staphylococcus aureus is the predominant pathogen in nonlimb-threatening foot infections, particularly in diabetic patients who have not yet received antimicrobial therapy (3–5). Although limb-threatening infections in the diabetic individual are frequently polymicrobial, *S. aureus*

is a major pathogen in these infections (6). Type 1 diabetic patients show more frequent colonization of the nose and skin by *S. aureus* than nondiabetic and non–insulin-dependent diabetic individuals (7–9). The emergence of *S. aureus* strains resistant to multiple antibiotics has made the treatment of staphylococcal infections especially problematic. Methicillin-resistant *S. aureus* strains have become increasingly prevalent among both nosocomial and community-acquired infections (10,11). Recently, *S. aureus* isolates resistant to vancomycin, one of the antibiotics of last resort, were recovered from patients with chronic foot ulcers (12,13). Although the increased susceptibility of diabetic patients to bacterial infections is well established, the chronicity associated with these infections remains poorly understood.

Many investigations have focused on the nonobese diabetic (NOD) mouse as a model for analyzing the development of type 1 diabetes. We describe an NOD mouse model of distal lower-extremity staphylococcal infection and identify several host factors that increase the susceptibility of diabetic mice to *S. aureus* infection. This animal model may allow the identification of additional defects in the innate immune response of diabetic humans that result in recalcitrant foot infections.

RESEARCH DESIGN AND METHODS

NOD mice were derived by selective inbreeding of a single female glucosuric mouse from a substrain of ICR mice (14). Female NOD mice spontaneously develop type 1 diabetes between 15 and 30 weeks of age (15), and by 20 weeks of age, 70–80% of females become diabetic. We obtained C57Bl/6 and NOD mice from The Jackson Laboratories (Bar Harbor, ME) and bred NOD mice in our facility. The mice were housed in a modified barrier facility under viral antibody-free conditions and were fed an autoclaved diet. Animal care was in accordance with guidelines set forth by Harvard Medical School. Blood glucose levels were tested with glucosrips (Bayer, Elkhart, IN), and ketone levels were tested with Acetest tablets (Bayer). Nondiabetic mice were normoglycemic with blood glucose levels ranging from 4 to 7 mmol/l. Diabetic mice had negative or low blood acetone levels, normal body mass, and blood glycemic levels ≥19 mmol/l (342 mg/dl) for 3–5 days before bacterial challenge.

Mouse model of *S. aureus* hindpaw infection. *S. aureus* strain PS80 (streptomycin resistant) is a capsular serotype 8 isolate that is virulent in a rat model of staphylococcal abscess formation. Capsule type 8 strains comprise ~50% of *S. aureus* isolates recovered from a variety of human infections (16,17). Mice were anesthetized systemically with 100 mg/kg ketamine and 10 mg/kg xylazine. The plantar aspect of the left hindpaw was disinfected with povidone-iodine and 70% ethanol, and a 10-μl suspension of *S. aureus* was injected at a depth of 2–4 mm into the plantar-proximal aspect of the hindpaw with a 29 1/2-G hypodermic needle and syringe. Groups of animals were killed between 1 and 10 days after bacterial challenge, and the hindpaws were amputated and defleshed. The tissues were weighed and homogenized, and dilutions of the homogenates were plated quantitatively onto tryptic soy agar medium. Bacterial colonies recovered from the tissues were replica plated to media supplemented with 500 μg/ml streptomycin to verify their identity with the *S. aureus* PS80 inoculum.

Some of the diabetic NOD mice received sustained insulin from LinBit

implants (LinShin, ON, Canada) that were placed subcutaneously in the abdomen. The release rate was ~0.1 units/day insulin, and the mice received either one or two LinBits. Blood glucose levels were evaluated for up to 2 weeks after implantation to verify normoglycemia before the mice were challenged with *S. aureus*.

Histologic examination of hindpaw tissues. Histologic analyses were performed on infected hindpaw tissues from nondiabetic and diabetic NOD mice that were killed at 6, 24, 48, or 72 h, or 5 days after inoculation with 10^6 colony-forming units (CFU) *S. aureus*. Hindpaw tissues were excised and fixed in formaldehyde. The sections were decalcified, embedded in paraffin, and stained with hematoxylin and eosin. Tissue sections were evaluated at magnifications of $100\times$ and $400\times$ by both authors in a blinded fashion. A myeloperoxidase assay (18) was used to quantify neutrophil accumulation in the hindpaw tissues.

Cytokine detection and measurement. Nondiabetic and diabetic NOD mice were inoculated with either 10^6 CFU *S. aureus* or sham inoculated with PBS. Infected animals were killed 6–48 h after bacterial challenge. The plantar musculature of the hindpaw was removed from the bone, weighed, and placed in 500 μl lysate buffer containing 1% Nonidet P40, 500 mmol/l NaCl, 50 mmol/l HEPES, and a mammalian protease inhibitor (P-8340; Sigma). The tissues were homogenized and stored at −70°C. Protein concentrations in soluble extracts from the tissues were determined by the Bradford method (19). Cytokine profiles in tissue extracts (diluted to 0.855 mg/ml protein) were evaluated with a mouse cytokine antibody array kit (Raybio 2.1; Raybiotech, Norcross, GA) that simultaneously detects 32 cytokines. Keratinocyte-derived chemokine (KC) and macrophage inflammatory protein 2 (MIP-2) concentrations were quantified in dilutions of the tissue extracts by cytokine-specific assays (Quantikine Immunoassay; R&D Systems, Minneapolis, MN).

Blood killing assays. Heparinized blood was collected from the tail vein of age-matched nondiabetic and diabetic NOD mice. Blood killing assays were performed in polypropylene tubes containing 200 μl mouse blood and 100 μl *S. aureus* to yield a final concentration of 10^5 CFU/ml. The samples were incubated at 37°C on a rotator, and aliquots were removed for quantitative culture after 0, 60, and 120 min. The data were expressed as percent reduction (or increase) from the initial inoculum (10^5 CFU/ml). For certain experiments, blood from nondiabetic mice was supplemented with α-D-glucose to yield final concentrations of 22 or 33 mmol/l. The glucose-blood mixture was incubated at 37°C for up to 3 h before the addition of *S. aureus*. Serum antibodies to the type 8 capsular polysaccharide were determined by enzyme-linked immunosorbent assay (20). Total complement activity (CH50) in mouse serum was assessed with a hemolytic assay using antibody-coated rabbit erythrocytes (Diamedix, Miami, FL). The C3 activity of NOD mouse serum was assessed by its ability to reconstitute the lytic potential of serum from C3-deficient mice (provided by Michael Carroll).

Functional analyses of mouse leukocytes. The respiratory burst of mouse leukocytes was evaluated by the oxidation of dihydrorhodamine 123 (DHR; Sigma). This dye diffuses inside the leukocytes and is colorless in its reduced form. When the dye is oxidized, it fluoresces and can be detected by flow cytometry. Heparinized blood (100 μl) was incubated for 20 min with 11 μmol/l DHR before the addition of 10 μl minimum essential medium (MEM; Invitrogen, Grand Island, NY) with 1% BSA (Sigma), 10^7 CFU *S. aureus*, or 1 μmol/l phorbol 12-myristate 13-acetate (PMA; Sigma). The samples were incubated for 30 or 60 min on a rotator at 37°C before harvesting by centrifugation. The erythrocytes were lysed in 0.2% NaCl followed by hypertonic rescue of the leukocytes with 1.6% NaCl. The leukocyte samples were washed twice in PBS/BSA and analyzed by FACScan cytometry (MoFlo High-Performance Cell Sorter; DakoCytomation, Fort Collins, CO). The samples were gated on the neutrophil population by forward and side scatter, and DakoCytomation software (Summit; Fort Collins, CO) was used for analysis of the data.

Statistical analyses. Comparisons between groups were made with the unpaired Student's *t* test or the Student's *t* test with Welch correction for unpaired samples (Instat 2.0; Graphpad Software, San Diego, CA). *P* values <0.05 were considered significant.

RESULTS

Hindpaw model of *S. aureus* infection. Preliminary experiments were aimed at developing a hindpaw model of *S. aureus* infection in nondiabetic C57Bl/6 mice. The strain PS80 inoculum was titrated from 10^8 to 10^4 CFU *S. aureus* per mouse. Animals inoculated with $<10^5$ CFU *S.aureus* resolved the infection by 5 days, whereas mice challenged with $\geq10^6$ CFU developed an infection that peaked 3–5 days postinoculation (data not shown). Five
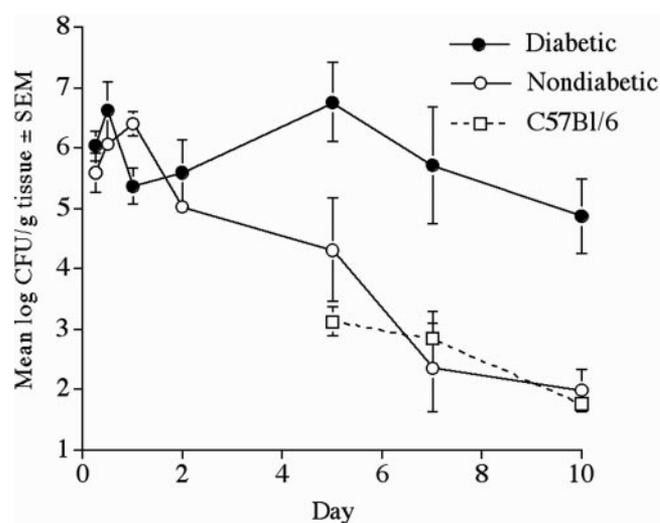

FIG. 1. Results of quantitative cultures performed on the hindpaws of age-matched diabetic NOD, nondiabetic NOD, and C57Bl/6 mice challenged with 10^6 CFU *S. aureus*. Each data point represents the geometric mean (±SE) from groups of three to nine mice killed at each time point. The lower limit of detection by culture was 1.77 log CFU *S. aureus*/g tissue.

days after inoculation with ~10^6 CFU *S. aureus*, the bacterial burden in the hindpaw tissues of seven mice 12–13 weeks of age was $\log_{10} 5.24 \pm 0.74$ CFU *S. aureus*. This value was significantly ($P = 0.0019$) greater than the bacterial burden ($\log_{10} 2.35 \pm 0.25$ CFU *S. aureus*) in the hindpaws of eight mice that were 7–8 weeks of age. Thus, mature C57Bl/6 mice were more susceptible to infection than the younger animals. All of the animals gradually cleared the infection over 15 days.

Subsequent experiments were performed to evaluate whether diabetic mice were more susceptible to staphylococcal infections than nondiabetic animals. In our NOD mouse colony, the majority of female mice develop diabetes between 15 and 24 weeks of age. We selected age-matched animals from the colony to include both diabetic and nondiabetic mice, and these animals were inoculated with ~10^6 CFU *S. aureus* PS80. Groups of animals were killed at various time points to evaluate the bacterial burden in the hindpaw tissues. Greater numbers of *S. aureus* were recovered from the infected tissues of diabetic mice on days 5 ($P = 0.035$), 7 ($P = 0.053$), and 10 ($P = 0.0095$) compared with nondiabetic NOD animals. The infection remained localized, and none of the animals developed bacteremia. The nondiabetic mice cleared the infection by day 10, whereas ~10^5 CFU *S. aureus*/g tissue persisted in the hindpaws of diabetic NOD animals. Age-matched C57Bl/6 mice were tested for comparison, and these mice showed a course of infection similar to that of nondiabetic NOD mice (Fig. 1).

Effects of hyperglycemia on *S. aureus* infection. To assess whether glycemic control would reduce the susceptibility of diabetic mice to staphylococcal infection, some diabetic mice received sustained insulin treatment from LinBit implants that were placed subcutaneously. The mice achieved normoglycemia 2–7 days after implantation, and they were inoculated with *S. aureus* 3–7 days later. As shown in Table 1, the insulin-controlled diabetic NOD mice had significantly fewer ($P = 0.027$) *S. aureus* recovered from their hindpaw tissues than diabetic animals 5 days after bacterial challenge.

TABLE 1
Quantitative culture results of hindpaw tissues of diabetic and insulin-controlled diabetic NOD mice challenged with 9×10^5 CFU and euthanized 5 days later

Mice	n	Blood glucose (mmol/l)	Log CFU per g tissue	P value
Diabetic	9	19 to ≥29	6.76 ± 0.65	0.027
Insulin-controlled diabetic*	3	6–9	3.47 ± 1.00	

*Insulin in the form of a sustained-release pellet was implanted subcutaneously in diabetic NOD mice.

Histopathology. Histologic tissue sections were prepared from infected hindpaws of nondiabetic and diabetic NOD mice that were killed after 6 h or up to 5 days following challenge with 10^6 CFU *S. aureus*. Polymorphonuclear leukocyte (PMN) infiltration 6 h after inoculation was more prominent in the hindpaw tissue from nondiabetic mice compared with diabetic animals (Fig. 2*A* and *B*). To quantify differences in PMN infiltration, we performed myeloperoxidase assays. Six of seven tissue samples excised from infected animals at the 6-h time point showed minimal myeloperoxidase activity. However, hindpaw tissues that were excised after 12 h from nondiabetic animals had significantly ($P = 0.0203$) greater myeloperoxidase activity (3.42 ± 0.48 units/g) than tissues from diabetic animals (0.10 ± 0.07 units/g) harvested at the same time point.

An inflammatory response with marked PMN infiltration was noted at 24 h in the infected hindpaw tissues from both nondiabetic and diabetic NOD mice (Fig. 2*C* and *D*).

Similar findings were observed 48 and 72 h after bacterial challenge (not shown). We observed more PMNs on day 5 in the hindpaw tissues of diabetic mice compared with the nondiabetic animals (not shown), but this was consistent with the greater bacterial burden in diabetic mice at this time point (Fig. 1).

Cytokine detection in infected hindpaws. To determine whether cytokine production in response to infection was dysregulated in diabetic NOD mice, we performed preliminary experiments with protein microarray kits that detected 32 different cytokines. Nine nondiabetic and nine diabetic NOD mice were inoculated with ~10^6 CFU *S. aureus* and killed 6–48 h later. Hindpaw tissue extracts from each animal and from sham-inoculated animals were diluted to the same protein concentration and tested on individual microarray membranes. Most of the 32 cytokines identified by the protein microarray kit, including tumor necrosis factor-α, interferon-γ, and interleukin-12, were below the level of assay detection (~10 pg/ml) in infected tissues from both groups of animals. Interleukin-6 levels were minimal in the infected mice. Consistent with their role in attracting neutrophils to infectious foci, the chemokines KC and MIP-2 gave detectable signals in tissues from infected mice but not from sham-inoculated animals. As shown in Fig. 3*A*, the signal intensities for KC and MIP-2 at 12-h postinoculation were greater in the tissue extracts from nondiabetic than from diabetic mice. To quantify KC and MIP-2 levels, we performed immunoassays on the hindpaw extracts from infected mice. As shown in Fig. 3*B*, the concentrations of KC in the tissue extracts from nondiabetic animals were higher than those of diabetic mice at 6 ($P = 0.0004$) and 12 ($P = 0.0664$) h
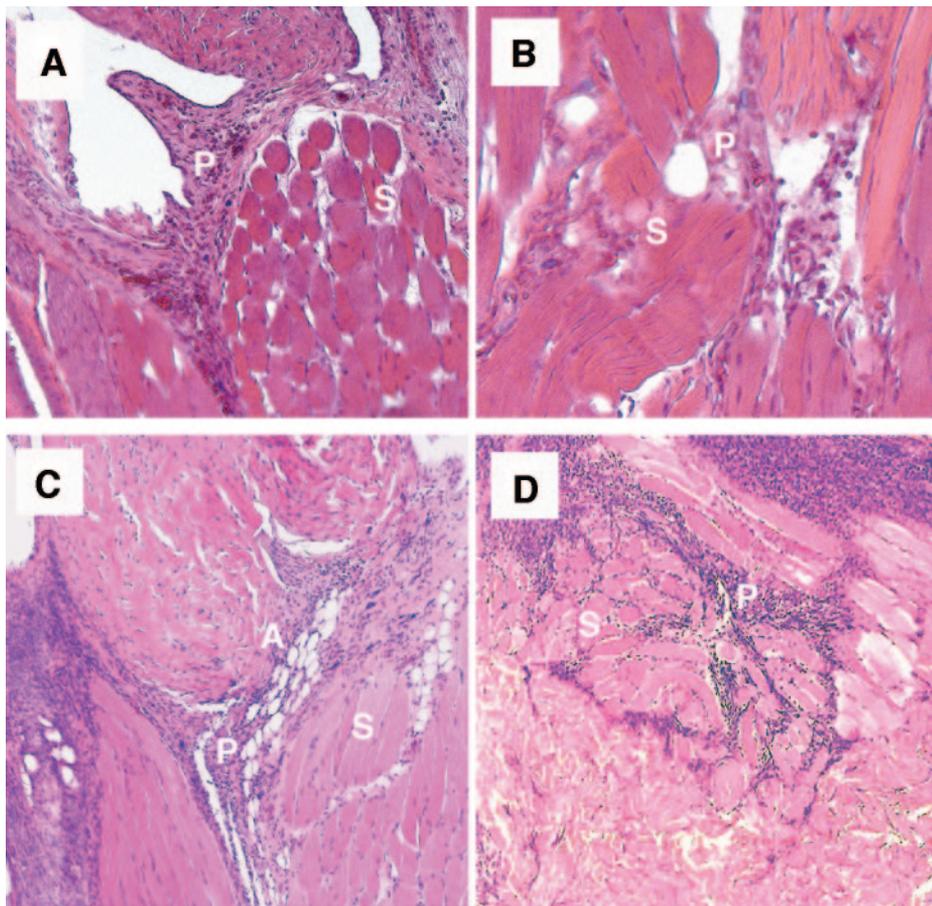

FIG. 2. Hematoxylin and eosin–stained sections (100×, original magnification) from hindpaw tissues of nondiabetic (*A* and *C*) and diabetic (*B* and *D*) NOD mice killed 6 h (*A* and *B*) or 24 h (*C* and *D*) after challenge with 10^6 CFU *S. aureus*. A paucity of PMNs is seen in the diabetic hindpaw tissues at 6 h compared with the nondiabetic tissues. PMNs are seen infiltrating the muscle tissues in both samples at 24 h. A, adipose tissue; P, PMNs; S, skeletal muscle.
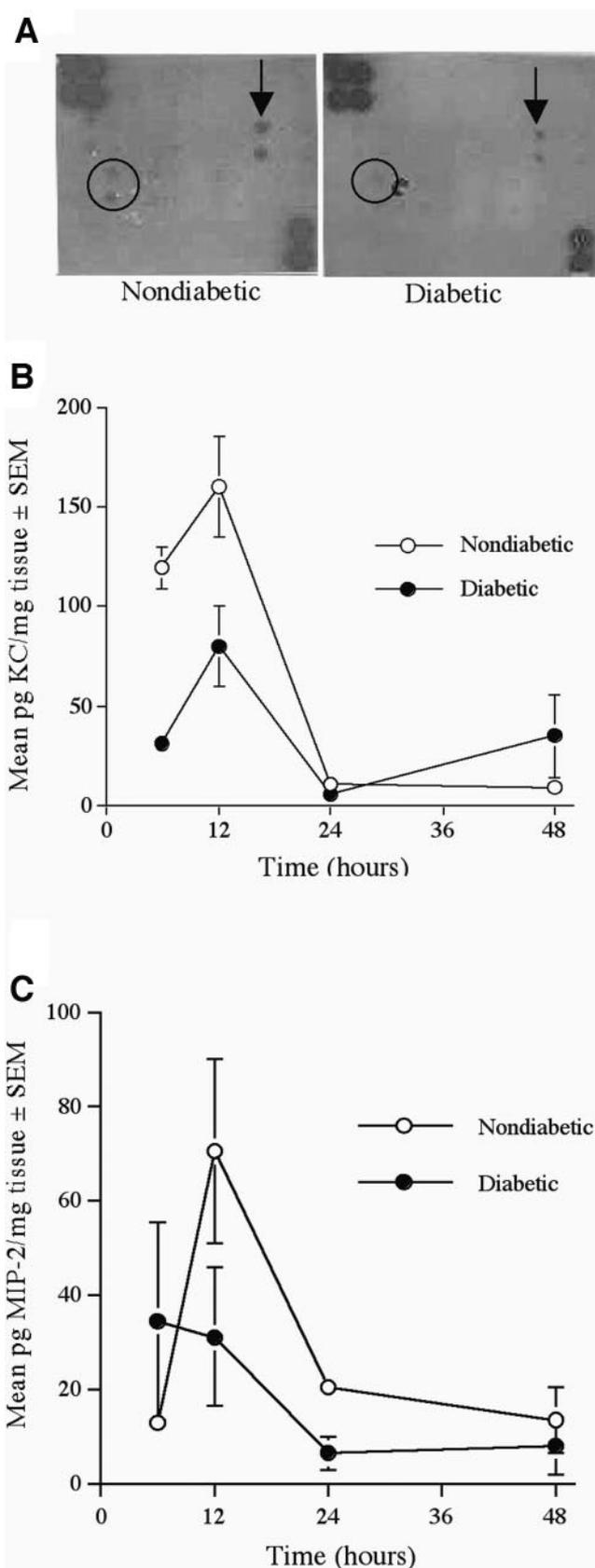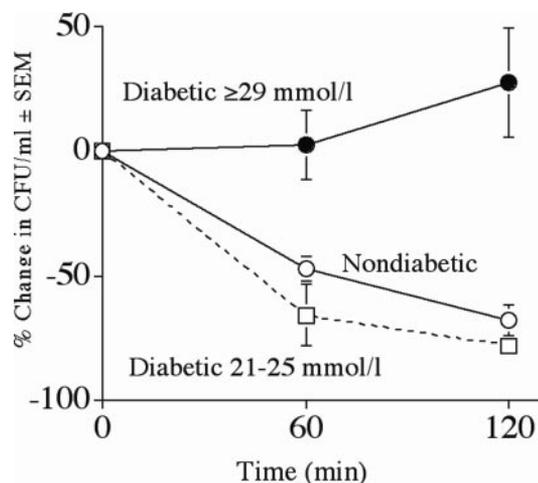
FIG. 4. Bactericidal activity of blood from nondiabetic and diabetic mice against 10⁵ CFU *S. aureus*. The data represent the mean values from 11 different assays performed on the blood of 6 diabetic and 11 nondiabetic mice. ●, diabetic mice (blood glucose ≥29 mmol/l); ○, nondiabetic mice; □, diabetic mice (blood glucose 21–25 mmol/l).

after inoculations with *S. aureus*. The sham-inoculated mice had KC levels ≤5 pg/mg tissue 12 h after bacterial challenge. The tissue concentrations of MIP-2 in nondiabetic mice were also higher than those of diabetic animals (Fig. 3C), but the differences were only significant ($P = 0.0447$) at the 24-h time point.

Blood killing assays. Neutrophils from diabetic patients have been shown to exhibit impaired bactericidal activity (21–23). To determine whether leukocytes from diabetic NOD mice would show a similar defect, we performed *S. aureus* killing assays with mouse blood. As shown in Fig. 4, blood from nondiabetic mice killed ~68% of the bacterial inoculum after a 2-h incubation at 37°C. Similar levels of *S. aureus* killing were observed in the blood from ICR (24) and C56Bl/6 mice (data not shown). The degree of bactericidal activity in the blood from individual diabetic NOD mice correlated with their blood glucose level. Blood from diabetic animals with glucose levels between 21 and 25 mmol/l killed *S. aureus* as well as blood from nondiabetic animals. However, *S. aureus* multiplied in the blood from animals with blood glucose levels ≥29 mmol/l (Fig. 4). We had three diabetic mice with blood glucose levels of 21, 22, and 24 mmol/l; their blood killed 78, 81, and 74%, respectively, of the *S. aureus* inoculum. *S. aureus* multiplied in the blood of the same three animals 3–9 days later when their blood glucose concentrations were >29 mmol/l. However, when we preincubated the blood from nondiabetic NOD mice with 22 or 33 mmol/l α-D-glucose for up to 3 h at 37°C, phagocytic killing was not impaired (data not shown). Differences in anticapsular antibody levels in the sera of diabetic vs. nondiabetic NOD mice could influence phagocytic killing of *S. aureus* in the whole-blood killing assays (24,25). However, when we tested sera from 17 nondiabetic and 5 diabetic animals (16–19 weeks of age), none of these naïve animals had detectable antibody levels to the type 8 capsular polysaccharide, as measured by enzyme-linked immunosorbent assay.

FIG. 3. Protein microarray membranes (A) were incubated with 12-h tissue extracts excised from nondiabetic or diabetic mice. Positive signals in the *upper left* and *lower right* represent positive controls. Signals from KC (↓) and MIP-2 (○) are shown in duplicate and appear enhanced in the sample from the nondiabetic NOD mouse. KC (*B*) and MIP-2 (*C*) levels were quantitated in tissue extracts from groups of two to six nondiabetic or diabetic mice that were killed 6–48 h after inoculation with *S. aureus*. KC levels in diabetic mice were diminished compared with nondiabetic animals after 6 ($P = 0.0004$) and 12 ($P = 0.0664$) h. MIP-2 concentrations in tissues from diabetic mice were significantly ($P = 0.0447$) lower than nondiabetic mice at the 24-h time point.
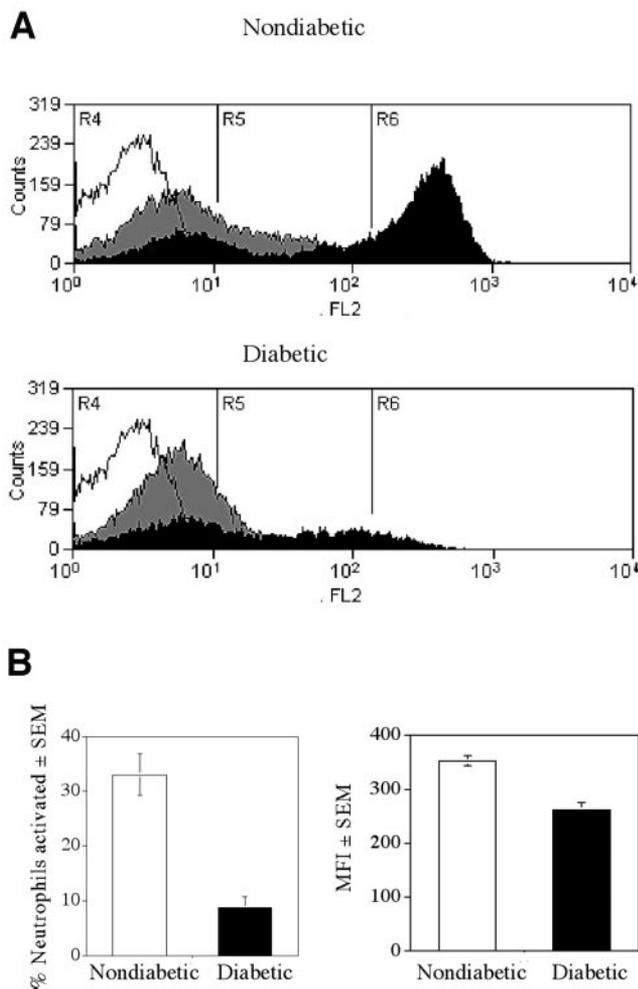
A

Nondiabetic

Diabetic

B

FIG. 5. Differential respiratory burst activity of leukocytes from nondiabetic and diabetic NOD mice. *A*: Representative results from assays performed with NOD mouse blood incubated for 30 min with *S. aureus*. Unshaded peaks represent unstained cells, gray peaks represent leukocytes stimulated with MEM, and black peaks represent cells stimulated with *S. aureus*. *B*: Summary of flow cytometry experiments on leukocytes from nondiabetic and diabetic NOD mice incubated with *S. aureus* for 30 min. The mean percent of stimulated cells and the mean fluorescence index for nondiabetic mice were significantly higher ($P < 0.0001$) than those for diabetic animals. The data represent the means of 21 respiratory burst assays performed on the bloods of eight nondiabetic and eight diabetic NOD mice.

NOD mice have a C5 deficiency (26) that is common to inbred mouse strains, and we have verified that blood from nondiabetic and diabetic NOD mice in our colony lack activity in a CH50 assay. Phagocytic clearance of *S. aureus* in vivo is dependent upon C3 (27), since C3b and iC3b are the principal complement opsonins important for uptake and killing of staphylococci by PMNs. Serum from both nondiabetic and diabetic mice was able to restore hemolytic activity to sera from C3-deficient mice (data not shown).

Functional analyses of mouse leukocytes. To determine whether the diminished bactericidal activity of blood from diabetic NOD mice could be attributed to impaired phagocyte function, respiratory burst assays were performed. Leukocytes from age-matched nondiabetic or diabetic animals were incubated with a stimulus (MEM, *S. aureus*, or PMA), and the oxidative burst was measured by flow cytometry. Figure 5*A* shows the results of a typical experiment wherein the R4 region was gated on unstained

cells, the R5 region was gated on cells stained with reduced DHR dye, and the R6 region was gated on cells that oxidized the dye. Neutrophils from the nondiabetic animal showed a marked shift in fluorescence in response to *S. aureus*, indicative of a robust respiratory burst. In contrast, neutrophils from the diabetic NOD mouse responded poorly to the *S. aureus* stimulus (Fig. 5*A*).

The data in Fig. 5*B* summarize the neutrophil responses of eight diabetic NOD mice that were tested in 18 different respiratory burst assays and compared with nondiabetic controls. Significantly fewer ($P < 0.0001$) neutrophils from diabetic mice responded to the *S. aureus* stimulus, and the magnitude of the response (as measured by the mean fluorescence index) was significantly lower ($P < 0.0001$) in the diabetic animals. The decreased respiratory burst seen in diabetic mice was more apparent at 30 min than 60 min (data not shown), which suggests that the oxidative burst is delayed in neutrophils from diabetic NOD mice in this model. Likewise, $42 \pm 5.9\%$ of leukocytes from three nondiabetic animals responded to PMA, whereas only $22 \pm 11\%$ of leukocytes from three diabetic mice responded ($P = 0.0848$).

DISCUSSION

Type 1 diabetes produces a ketotic-prone, insulin-dependent disease state in humans. Similarly, NOD animals develop spontaneous autoimmune diabetes and exhibit many immunologic and clinical similarities to patients with type 1 diabetes. The NOD mouse is a well-described model of type 1 diabetes, with early leukocytic infiltration of the islets of Langerhans, leading to overt diabetes between 15 and 30 weeks of age (28). The onset of diabetes is recognized by the appearance of glycosuria and by nonfasting plasma glucose levels ≥ 17 mmol/l. Glycosuria and hyperglycemia in NOD mice become progressively more severe over several weeks, at which time weight loss, polyuria, and polydipsia may occur.

Animal models of type 1 diabetes have been used to characterize the immunologic mechanisms that lead to diabetes. The pathogenesis of diabetes complications such as chronic foot infections has received less attention. Diabetic patients often experience recalcitrant and debilitating staphylococcal foot infections, and this suggests that some of these individuals possess specific defects in the innate immune system that are poorly understood.

To address this common complication of diabetes, we developed a model of *S. aureus* infection in the hindpaw of NOD mice. The experimental infection was exacerbated in diabetic mice compared with nondiabetic mice, and the diabetic animals were unable to clear the infection over a 10-day period. Insulin-mediated control of glycemia in diabetic mice resulted in enhanced clearance of *S. aureus* from the hindpaw tissues, similar to that observed in nondiabetic and C57Bl/6 mice. The observed resistance of mice with glycemic control correlates with the fact that diabetic patients with well-controlled blood glucose are no more susceptible to bacterial infections than are healthy individuals.

An important component of the innate host defense against bacterial infection is a brisk inflammatory response by "professional" phagocytes. PMNs are the primary inflammatory cells observed in acute infections. Chemokines, including KC and MIP-2, play an important role in the recruitment of neutrophils to the site of infection or trauma. In the early stages of the bacterial

infection (6–24 h), diabetic NOD mice showed decreased concentrations of KC and MIP-2 in the infected hindpaw compared with nondiabetic mice. Thus, fewer PMNs may be recruited to infected tissues of diabetic mice, and these PMNs may not be optimally activated. The low KC and MIP-2 levels in tissues from diabetic mice correlated with the diminished inflammatory response that we observed 6–12 h after bacterial challenge. PMN infiltration assessed by histology appeared similar in the nondiabetic and diabetic animals by 24 h. This delay in the innate immune response could result in the invading bacteria gaining a foothold in the damaged tissues of the diabetic host.

Defects in the bactericidal activity of PMNs from type 1 diabetic individuals have been reported (21–23,29–32). Diabetic patients with impaired phagocyte function and respiratory burst show improved neutrophil activity following glycemic control. Similar to humans with uncontrolled diabetes, NOD mice with blood glucose levels ≥29 mmol/l exhibited poor leukocyte bactericidal activity. However, blood from diabetic mice with glucose levels <25 mmol/l showed good killing activity against *S. aureus*. Multiple assays performed on blood from three diabetic mice showed decreasing phagocytic activity as blood glucose concentrations increased over time. However, additional studies are warranted to discern whether there is an actual threshold of hyperglycemia at which *S. aureus* killing is impaired. In vitro experiments wherein blood from nondiabetic mice was preincubated with glucose (22 or 33 mmol/l) showed no impairment in bactericidal activity.

Other investigators have analyzed the effect of a hyperglycemic state on neutrophil function by adding glucose to nondiabetic blood. These studies have yielded variable results ranging from decreased phagocytic killing and respiratory burst to no effect on neutrophil function (32–34). It is likely that diminished phagocyte function requires additional physiologic factors, e.g., glycosylation of proteins and oxidative stress, rather than merely the presence of excess glucose to simulate an in vivo hyperglycemic environment.

Proper glycemic control (blood glucose <11.1 mmol/l) in diabetic patients can reduce the progression of microvascular complications, e.g., retinopathy, nephropathy, and neuropathy (35). Well-controlled plasma glucose not only reduces acute complications, such as diabetic keto-acidosis and hyperosmolar hyperglycemic nonketotic syndrome, but may also prevent the onset and progression of chronic complications such as wound healing impairment and degenerative changes in the ankle and foot (6,36).

The poor bactericidal activity of neutrophils from diabetic hosts correlates with defects in the respiratory burst of these cells. PMNs from diabetic patients have shown decreased oxidative potential upon stimulation with zymosan (37,38). Likewise, we showed that leukocytes from diabetic NOD mice had a decreased respiratory burst compared with nondiabetic animals after a 30-min incubation with whole *S. aureus* cells or PMA. PMA is an agonist of PMN oxidative burst and works by directly activating protein kinase C. Studies with animal models of type 1 diabetes other than NOD mice demonstrate variable results with regard to leukocytic respiratory burst (39,40).

Although the NOD mouse has provided important new immunogenetic and pathophysiologic insights into autoimmune disease, our hindpaw infection model has inherent limitations. Diabetic humans often present with distal peripheral neuropathy, an important initiating factor for foot ulceration and infection. The absence of peripheral neuropathy in NOD mice precludes the evaluation of ulceration and wound healing impairment in this animal model. Moreover, vasculopathy and nephropathy have not been reported in NOD mice, and such pathology can impede wound healing and exacerbate the development of chronic foot infections.

Our diabetic mice had glucose levels ≥19 mmol/l and thus represent uncontrolled diabetes, a state that is not typically observed in clinically compliant patients who regularly monitor their blood glucose and adhere to insulin regimens. Moreover, the hyperglycemia present in NOD mice is not chronic as it is in humans. In the absence of treatment with insulin, our mice become emaciated and moribund within 3 weeks of the onset of hyperglycemia. Thus, it is not feasible for us to perform glycosylated hemoglobin tests with NOD mice and monitor the effects of long-term hyperglycemia on chronic infection.

In summary, the NOD mouse may prove to be an important tool to dissect the immunologic mechanisms behind increased susceptibility to bacterial infection in the diabetic host. Hyperglycemia is clearly an important contributing factor. Diabetic NOD mice show heightened susceptibility to infection, diminished CXC chemokine production, and decreased neutrophil function in response to *S. aureus*. The role of chemokines and cytokines in modulating neutrophil function in the diabetic mouse is currently under investigation in our laboratory, and these studies may shed light on immunologic mediators important in resolving diabetic foot infections.

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