The Journal of Immunology
RESEARCH ARTICLE | APRIL 01 2000

Survival of *Staphylococcus aureus* Inside Neutrophils Contributes to Infection [1] FREE

Hattie D. Gresham; ... et. al

J Immunol (2000) 164 (7): 3713–3722.
https://doi.org/10.4049/jimmunol.164.7.3713

Survival of *Staphylococcus aureus* Inside Neutrophils Contributes to Infection[1]

Hattie D. Gresham,[2]*† Jon H. Lowrance,*† Tony E. Caver,*† Bridget S. Wilson,‡ Ambrose L. Cheung,§ and Frederik P. Lindberg¶

Neutrophils have long been regarded as essential for host defense against *Staphylococcus aureus* infection. However, survival of the pathogen inside various cells, including phagocytes, has been proposed as a mechanism for persistence of this microorganism in certain infections. Therefore, we investigated whether survival of the pathogen inside polymorphonuclear neutrophils (PMN) contributes to the pathogenesis of *S. aureus* infection. Our data demonstrate that PMN isolated from the site of infection contain viable intracellular organisms and that these infected PMN are sufficient to establish infection in a naive animal. In addition, we show that limiting, but not ablating, PMN migration into the site of infection enhances host defense and that repletion of PMN, as well as promoting PMN influx by CXC chemokine administration, leads to decreased survival of the mice and an increased bacterial burden. Moreover, a global regulator mutant of *S. aureus* (*sar*−) that lacks the expression of several virulence factors is less able to survive and/or avoid clearance in the presence of PMN. These data suggest that the ability of *S. aureus* to exploit the inflammatory response of the host by surviving inside PMN is a virulence mechanism for this pathogen and that modulation of the inflammatory response is sufficient to significantly alter morbidity and mortality induced by *S. aureus* infection. *The Journal of Immunology,* 2000, 164: 3713–3722.

Staphylococcus aureus is a major human pathogen causing significant morbidity and mortality in both community- and hospital-acquired infections (1). It causes a diverse array of infections ranging from relatively minor skin and wound infections to more serious and life-threatening diseases such as pneumonia, endocarditis, osteomyelitis, arthritis, and sepsis. Concern over the emergence of multidrug-resistant strains has renewed interest in understanding the virulence mechanisms of this pathogen at the molecular level and in elucidating host defense elements that either provide protection from or limit infection (2–7). Polymorphonuclear neutrophils (PMN)[3] have long been thought to provide significant host defense against *S. aureus* infection primarily because patients who are neutropenic or who have congenital or acquired defects in PMN function are more susceptible to infection with this pathogen. This essential role for PMN in an experimental model of *S. aureus*-induced septic arthritis was recently confirmed by depleting mice of granulocytes before the establishment of infection (8). In this study, the total ablation of PMN resulted in decreased survival of the mice and increased bacteremia, suggesting that some numbers of PMN are necessary to provide protection against this pathogen. However, in our studies of TGF-β-mediated defects in PMN function and host defense against *S. aureus* infection in autoimmune mice, we noted that decreased survival of the mice correlated with excessive numbers of PMN and an increased bacterial burden at the site of infection (9, 10). These data raised the question as to whether the increased numbers of PMN actually contributed to the increased number of organisms. Taken together, these two sets of data imply that PMN could have both a protective and a deleterious role in *S. aureus* infection.

The possibility that phagocytes, particularly PMN, could facilitate *S. aureus* infection has been raised by other investigators (11–15). In vitro studies from the 1950s and 1960s demonstrated that pathogenic strains of *S. aureus* could survive for long periods of time inside both PMN and monocytes isolated from different animals and humans (12–14). These studies led investigators at the time to speculate that both intracellular survival and extracellular multiplication play important roles in the pathogenesis of *S. aureus* infections (11). In this regard, recent experiments assessing invasion and intracellular survival of *S. aureus* in endothelial cells (16), epithelial cells (17), and osteoblasts (18) have suggested that intracellular survival could contribute to the persistence of the pathogen in *S. aureus*-induced endocarditis, bovine mastitis, and osteomyelitis. Although these in vitro studies speculate on the adaptive advantage of intracellular survival in nonprofessional phagocytes, no studies have assessed whether survival of *S. aureus* inside PMN occurs in vivo and whether this can promote infection.

Therefore, the purpose of this work is to test the hypothesis that survival of *S. aureus* inside PMN contributes to the pathogenesis of this infection. As with earlier in vitro studies (11–15), we found that PMN isolated from the site of infection contained viable intracellular bacteria. Moreover, we show that the ability of *S. aureus* to exploit the host's inflammatory response is regulated by

*Research Service, Albuquerque Veterans Affairs Medical Center, Albuquerque, NM 87108; Departments of †Molecular Genetics and Microbiology and ‡Pathology, University of New Mexico, Albuquerque, NM 87131; §Department of Microbiology, Dartmouth Medical School, Hanover, NH 03755; and ¶Departments of Infectious Diseases and Molecular Microbiology, Washington University School of Medicine, St. Louis, MO 63110

Received for publication October 25, 1999. Accepted for publication January 21, 2000.

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.

[1] This work was supported by the Medical Research Service of the Department of Veterans Affairs (H.D.G.), the Dedicated Research Funds of the University of New Mexico School of Medicine (H.D.G.), the American Arthritis Foundation (F.P.L.), and National Institutes of Health Grants GM 57573 (F.P.L.), AI 37142 (A.L.C.), and AI 30061 (A.L.C.).

[2] Address correspondence and reprint requests to Dr. Hattie D. Gresham, Department of Molecular Genetics and Microbiology, Room 379, Basic Biomedical Science Building, 915 Camino de Salud, University of New Mexico, Albuquerque, NM 87131-5276. E-mail address: hgresham@salud.unm.edu

[3] Abbreviations used in this paper: PMN, polymorphonuclear neutrophils; IAP, integrin-associate protein; HSA, human serum albumin; MIP, macrophage-inflammatory protein.

0022-1767/00/$02.00

sar, a global regulator of multiple virulence factors in this organism (2). These data demonstrate that although necessary for infection control, appropriately stimulated PMN can create an environment that promotes the intracellular survival of this pathogen to the detriment of the host.

Materials and Methods

Mice

C57BL/6J and BALB/cByJ mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice deficient in integrin-associate protein (IAP, CD47) were backcrossed onto either C57BL/6J or BALB/cJ strains as described (19). All studies were approved by the Institutional Animal Use Committees. For infection studies, mice were age and gender matched. To obtain bone marrow leukocytes, femurs were removed aseptically and flushed with HBSS/0.2% human serum albumin (HSA). PMN were isolated from bone marrow leukocytes using NIM2 isolation media (Cardinal Associates, Santa Fe, NM) following the manufacturer's instructions and resuspended in endotoxin-free sterile saline (McGaw, Irvine, CA). The cells were 75–80% PMN as determined by staining with 0.1% cresyl violet.

Bacterial strains

S. aureus strain DB, a microencapsulated clinical blood isolate, and a *sar*$^-$ transposon-induced mutant of DB, 11D2, were characterized as described (2, 3, 7). 11D2 is significantly less virulent than DB in a rabbit model of *S. aureus*-induced endocarditis (3). RN6390, a prototypic, type 5 microencapsulated strain, was characterized as described (7). Strain SA1, an encapsulated and TSST-1-positive clinical isolate, was provided by Dr. J. Lee (Harvard University, Cambridge, MA) (20). Type III *Streptococcus pneumoniae* was purchased from the American Type Culture Collection (ATCC) (no. 10813). Bacteria were cultured on either blood agar or Columbia salt agar.

Lethal and sublethal infection models

S. aureus infection of mice was performed as described previously (9). Bacteria were adjusted to the desired inoculum spectrophotometrically before injection (OD_{620} = 1.6 for $\sim 1.0 \times 10^9$ CFU/ml), and the CFU were confirmed by serial dilution and culture on blood agar (Becton Dickinson, Cockeysville, MD). Survival in response to i.p. challenge and the number of WBC per ml, percentage of PMN, number of PMN per ml, and the $\log_{10}$ CFU/ml of a 5-ml peritoneal lavage (HBSS with 0.2% HSA and 5 mM EDTA) obtained 24 h after i.p. challenge (both lethal and sublethal CFUs) were performed as described (9). The challenge CFUs are given in the figure legends. Mice were sacrificed when moribund as assessed by decreased body temperature, impaired ambulation, the inability to remain upright, prolonged lethargy, and prolonged inappetance. At the lethal dose, the moribund condition was reached by 24–72 h.

Gentamicin protection assay of S. aureus *associated with PMN in vivo*

To determine whether viable *S. aureus* organisms associated with PMN were intracellular, we performed a gentamicin protection assay on the extracellular and PMN-associated bacteria in the peritoneal lavage obtained from mice 24 h after i.p. challenge. Gentamicin kills *S. aureus* but is limited in its ability to gain access to intracellular organisms. The peritoneal lavages from several infected mice were combined, and the PMN were purified by depleting lymphocytes and macrophages by negative selection as described (10). The Abs used for depletion were 30H12 (rat IgG2b anti-mouse Thy1.2), GK1.5 (rat IgG2b anti-mouse CD4), and F4/80 (rat IgG2b anti-mouse macrophage). Ab-coated cells were incubated with 0.4 ml goat-anti-rat IgG magnetic particles (PerSeptive Diagnostics, Cambridge, MA) in cold HBSS buffer for 20 min on ice. The percentage of PMN in the peritoneal exudate was increased from ~82% to ~98% depending on the experiment, and PMN viability was >95%. Aliquots were taken from both the extracellular supernatant and the PMN suspension to assess $\log_{10}$ CFU before incubation with 100 μg/ml gentamicin at 37°C. After 2 h, the PMN and the extracellular bacteria were washed twice to remove the gentamicin and resuspended to the original volume of the lavage; aliquots were taken to assess the $\log_{10}$ CFU. To ensure that PMN-associated bacteria were capable of being killed by an antibiotic, the cell pellets were incubated with 1 mg/ml rifampin. This treatment significantly reduced the PMN-associated CFU.

Use of PMN containing viable S. aureus *to establish infection*

To ascertain whether PMN containing viable *S. aureus* can contribute to infection, PMN were purified from peritoneal lavages 24 h after infection with 1×10^6 SA1, treated with 100 μg/ml gentamicin for 2 h, and washed twice; an aliquot was taken to determine the CFU and was injected i.p. into wild-type mice. The number of PMN injected was determined to give $\sim 1 \times 10^6$ CFU of SA1. As a control, the equivalent number of uninfected PMN purified from the bone marrow were also injected i.p. After 24 h, a lavage was performed and the total number of PMN and the total $\log_{10}$ CFU were assessed.

Electron microscopy

To assess the intracellular locale of bacteria taken up by PMN, peritoneal exudate cells obtained 24 h after i.p. challenge with DB, the *sar*− mutant 11D2, or SA1 were analyzed by transmission electron microscopy. The cells were fixed as a pellet in 2% glutaraldehyde, osmicated in 2% osmium tetroxide, stained en bloc with 2% uranyl acetate, treated with propylene oxide, and Epon embedded. Ultrathin sections were placed on copper grids, stained with uranyl acetate and lead citrate, and viewed in an Hitachi 600 transmission electron microscope (21). Photographs of representative fields (two experiments for each strain) were analyzed, and the percentage of bacteria in large, spacious vacuoles, in tight phagosomes, and free in the cytoplasm (non-membrane bound) was quantified by two independent observers.

mAb treatment and chemokine administration

To partially or fully deplete PMN, we used administration of rat IgG2b anti-Gr-1 mAb RB6-8C5 as described by others (8, 22). The RB6-8C5 hybridoma was obtained from DNAX (Palo Alto, CA) and an isotype control (SFR3) which does not recognize murine cells was purchased from ATCC. The Abs were purified from culture supernatant by ammonium sulfate precipitation and protein G-Sepharose (mAb Trap GII; Pharmacia Biotech, Alameda, CA). Before injection, the purified Abs were subjected to buffer exchange against sterile endotoxin-free normal saline to ensure the absence of endotoxin as previously described (9). Mice were injected i.v. via the tail vein with 5–250 μg of mAb RB6 vs control SFR3 to assess the effect on PMN number and on the host response to infection. For partial depletion, 5–10 μg mAb RB6 per mouse consistently depleted 25–33% of bone marrow PMN. At this concentration, SFR3 had no effect. For full depletion, 100 μg or greater were required to deplete >90% of PMN for 72 h.

To promote PMN migration into the site of infection, we administered the murine CXC chemokines, macrophage-inflammatory protein (MIP)-2 and KC (R&D Systems, Minneapolis, MN). As a control we administered the murine CC chemokine MIP-1a. Recombinant MIP-2, KC, and MIP-1α were reconstituted in buffer containing 0.2% HSA and were stored at −70°C in sterile tubes precoated with 0.2% BSA-PBS. The stock was diluted with endotoxin-free sterile normal saline to deliver 400 ng in 100 μl vs a vehicle control of buffer diluted into saline. To assess CXC chemokine levels in lavage samples by ELISA (R&D), the samples were centrifuged, sterile-filtered, and stored at −70°C in tubes precoated with 0.2% BSA.

In vitro uptake of S. aureus *by human PMN*

To ascertain whether *S. aureus* can survive intracellularly in human PMN taken up in vitro, we incubated human PMN (1×10^7), isolated as described (23), with 1×10^8 bacteria (strains DB, 11D2, or SA1) in 1 ml of buffer (HBSS containing 0.5 mM Ca^{2+}, 2.5 mM Mg^{2+}, and 1% sterile, endotoxin-free HSA) in the presence or absence of various concentrations of recombinant human IL-8 or control chemokines (R&D Systems). The optimal concentration of IL-8 for uptake was 500 ng/ml. Minimal uptake of the bacteria was observed in the absence of IL-8. Controls included 500 ng/ml of the CC chemokine, MIP-1α, and 15 ng/ml phorbol dibutyrate. To control for the role of blood-derived opsonins, the effect of 10% autologous fresh human serum from a donor with agglutinating Ab against strain DB was also evaluated. After 4 h at 37°C, the extracellular and PMN-associated bacteria were subjected to gentamicin protection as described above. At this time, PMN viability was >95%.

Statistical analysis

Data are presented as the means ± SEM. Statistical analyses were determined by Fisher's exact test for the survival studies and by the Mann-Whitney *U* test for nonparametrics for all other analyses, using Statview for MacIntosh.

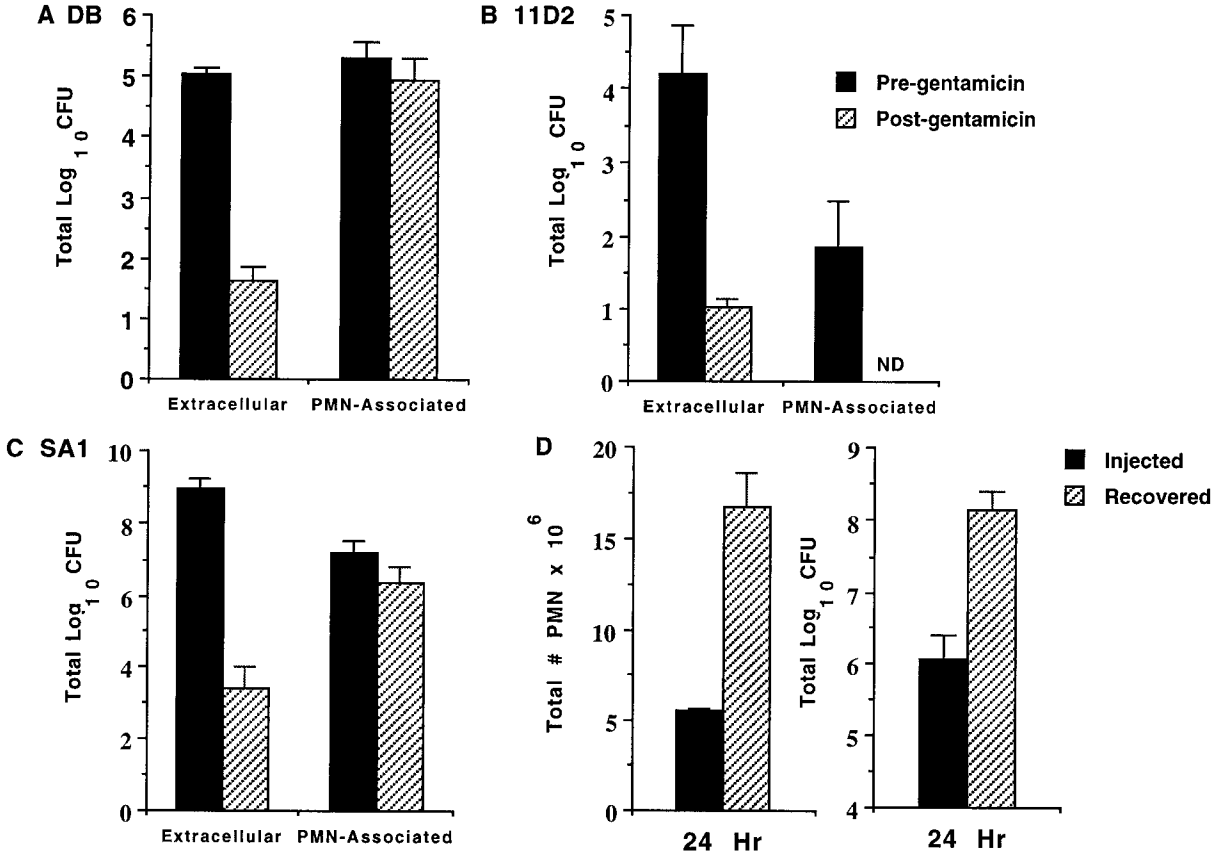

FIGURE 1. Effect of gentamicin treatment on the log CFU in the extracellular supernatant or associated with PMN in a peritoneal lavage performed 24 h after i.p. *S. aureus* challenge with 5×10^8 DB (*A*), 5×10^8 11D2 (*sar*− (*B*)), or 1–2 $\times$ 10^6 SA1 (*C*). Wild-type mice ($n = 3$, groups of 3 mice) were infected i.p., and a lavage was performed at 24 h. The extracellular supernatant was separated from the cell pellet by centrifugation. The cell pellet was enriched for PMN (to 98%) by depletion of macrophages and lymphocytes as described in *Materials and Methods*. Both the PMN and the extracellular supernatant were incubated with 100 μg/ml gentamicin to kill extracellular bacteria. After 2 h at 37°C, the CFU before and after gentamicin treatment was assessed by dilution and spread-plating. For all the strains, gentamicin treatment readily killed the extracellular bacteria. However, for the wild-type strains, but not for the *sar*− strain 11D2, gentamicin did not affect the number of PMN-associated CFU (ND = none detected). *D*, Effect of injection of PMN containing 1×10^6 CFU viable intracellular SA1 into the peritoneum of wild-type mice ($n = 3$) on the number of PMN and the bacterial burden in a lavage of the peritoneum performed 24 h after injection. The number of PMN and bacteria injected (■) is compared with the number recovered (▨). The number of PMN recovered and the total CFU were significantly increased ($p = 0.0044$ and 0.0082, respectively). PMN containing viable intracellular SA1 are sufficient to induce an influx of additional PMN and to establish an environment that significantly increases the growth of the pathogen.

Results

PMN isolated from the site of infection contain viable intracellular S. aureus *and are sufficient to establish infection in naive mice*

To assess whether viable *S. aureus* could be detected inside PMN isolated from a site of infection, we subjected both the PMN and the extracellular supernatant of peritoneal lavages obtained 24 h after i.p. challenge of wild-type mice to gentamicin treatment. *S. aureus* is susceptible to gentamicin, but because gentamicin does not readily cross plasma membranes, intracellular organisms are protected from optimal killing. For strain DB, a microencapsulated clinical blood isolate (2, 3), equivalent numbers of bacteria were extracellular and PMN-associated (Fig. 1*A*). However, the extracellular DB were readily killed by gentamicin whereas the PMN-associated organisms were not significantly affected, indicating that they remained viable inside the PMN (Fig. 1*A*). Equivalent data were obtained with an additional microencapsulated strain RN6390 as with DB (data not shown). If intracellular survival represents a virulence mechanism for *S. aureus*, then mutants with attenuated virulence should be less able to survive inside PMN. To address this, we examined a transposon-induced mutant of DB, 11D2, which has attenuated virulence in several animal models due to the loss of a regulatory locus, *sar*, which governs the synthesis and secretion of several virulence factors, including matrix protein binding adhesins (2, 7). The *sar*− mutant had significantly fewer organisms associated with PMN, and these were not protected from gentamicin killing (Fig. 1*B*). To assess whether this was a property of other Gram-positive bacteria, we challenged mice with 1×10^3 CFU type III *Streptococcus pneumoniae* and assessed whether PMN isolated from the site of infection contained viable bacteria. No gentamicin-resistant organisms were detected associated with PMN (data not shown). These data indicate that at 24 h after the initiation of infection, wild-type *S. aureus*, but no other Gram-positive bacteria, remain viable inside PMN and that their intracellular survival is regulated, at least in part, by the *sar* regulatory locus.

Because capsule production is thought to inhibit uptake of *S. aureus* by PMN and because DB is microencapsulated, we asked whether a fully encapsulated strain, SA1, would be equally taken up by PMN and survive intracellularly. Whereas the majority of SA1 were extracellular, $\sim 10^7$ CFU were PMN associated and resistant to gentamicin killing equivalent to what we observed with

DB (Fig. 1*C*). These data indicate that capsule type does not affect the ability of *S. aureus* to gain access to an environment inside PMN that permits its survival.

The above data indicated that *S. aureus* remain viable inside PMN; however, this viability could be transient. To assess whether these infected PMN are capable of transferring infection, we injected SA1-infected, gentamicin-treated PMN i.p. into naive mice. As shown in Fig. 1*D*, injection of PMN containing $\sim 10^6$ viable SA1 was sufficient to cause an additional influx of PMN ($p = 0.0044$) and result in significant growth of the pathogen ($p = 0.0082$) 24 h after administration of the cells. Injection of an equivalent number of uninfected PMN purified from the bone marrow (4.55×10^6 injected PMN) resulted in a reduction in the number of PMN present at 24 h ($0.79 \pm 0.09 \times 10^6$ recovered PMN, $n = 3$), indicating that the presence of uninfected PMN is not sufficient to recruit additional PMN. The bacterial burden in the lavage consisted of both extracellular and intracellular organisms (data not shown). These data demonstrate that intracellular *S. aureus* survive long enough inside PMN to transfer infection.

sar+ *and* sar− S. aureus *are in physically different intracellular vacuoles*

To examine the location of *S. aureus* inside PMN isolated from a site of infection, we performed transmission electron microscopy on PMN from peritoneal lavages taken 24 h after i.p. challenge with *sar*+ strains DB and SA1 and *sar*− 11D2. Intracellular bacteria were found in large vacuoles, "spacious phagosomes," (Fig. 2 *A* and *B*), free in the cytoplasm (Fig. 2*C*), or in small vacuoles, "tight phagosomes" (Fig. 2*D*). For both DB (Fig. 2*A*) and SA1 (Fig. 2*B*), the majority of the bacteria were in large vacuoles similar to spacious phagosomes created by macropinocytic uptake (24–26) (76.9 and 69.6%, respectively). A minority were in tight phagosomes (12.8% for DB and 21.7% for SA1) or free in the cytoplasm (10.3% for DB and 8.7% for SA1). Individual PMN often contained bacteria in all three intracellular locales (data not shown). In contrast, *sar*− 11D2 were primarily in tight phagosomes (97.2%) and rarely in large vacuoles (2.8%) or free in the cytoplasm (2.8%). Some of the wild-type bacteria were in membrane-bound vacuoles (Fig. 2*B*, black arrows) whereas others were in vacuoles where the membrane had either totally or partially degraded (Fig. 2, *A* and *B*, white arrows). This same phenomenon was recently observed with *S. aureus* taken up by epithelial cells in vitro and was thought to precede escape of the bacteria into the cytoplasm (17). These data indicate that *sar*+ strains, which are able to survive inside PMN, are primarily located in macropinosome-type organelles (spacious phagosomes) and that *sar*− strains, which do not survive inside PMN, are in small vacuoles with tightly apposed membranes (tight phagosomes). Therefore, *sar* regulates intracellular survival of *S. aureus* by influencing the intracellular locale of the internalized bacteria.

Limitation of PMN migration into the site of infection enhances survival and reduces the bacterial burden in S. aureus *infection*

If survival of *S. aureus* inside PMN represents a significant contribution to infection with this pathogen, then limiting access of the organism to this intracellular site should be beneficial. To test this hypothesis, we assessed survival in response to *S. aureus*-induced peritonitis and sepsis in IAP-deficient mice (19). IAP is a multiply membrane-spanning member of the Ig gene family (27), which modulates PMN migration across endothelial cells (28). We have published previously that IAP deficiency limits PMN migration in response to *E. coli*-induced peritonitis resulting in an increased bacterial burden and increased mortality of the mice (19). Therefore, if reducing the number of PMN in *S. aureus*-induced peritonitis and sepsis would be beneficial, then IAP-deficient mice should have enhanced host defense against *S. aureus*. To examine this, we injected $IAP^{+/+}$ wild-type and $IAP^{-/-}$ C57BL/6 mice with a lethal dose of SA1 ($1–2 \times 10^6$ CFU) into the peritoneum and assessed survival. In contrast to what we observed with *Escherichia coli* (19), $IAP^{-/-}$ mice had significantly enhanced survival in response to SA1-induced sepsis (Fig. 3*A*, $p = 0.0055$). To assess the host response to infection at a time when most wild-type mice become moribund, we measured the leukocyte number, including the number of PMN, and the bacterial burden at the site of infection 24 h after infection. $IAP^{-/-}$ mice had significantly fewer PMN ($p = 0.0045$) and an $\sim$1.5 log reduction ($p = 0.0044$) in CFU at the site of infection. The appearance of the $IAP^{-/-}$ mice also correlated with the increased survival and reduced bacterial burden; they were grooming themselves, eating, drinking, and were less lethargic than $IAP^{+/+}$ mice. These data indicate that reduced PMN migration into the site of *S. aureus* infection due to IAP deficiency results in enhanced survival of the host and a reduced bacterial burden.

PMN activation could be contributing to lethality of wild-type mice by causing excessive tissue injury and not by primarily affecting bacterial burden. Therefore, we wanted to ascertain

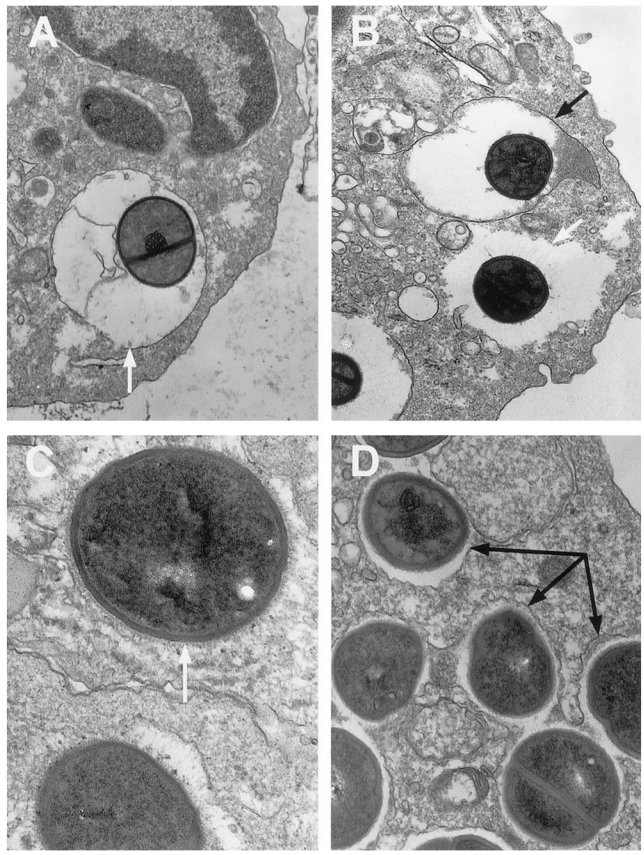

FIGURE 2. Electron microscopy of PMN from a peritoneal lavage obtained 24 h after i.p. challenge with $3–5 \times 10^8$ DB (*A* and *C*), 1×10^6 SA1 (*B*), or $3–5 \times 10^8$ 11D2 (*D*) (*sar*−). The PMN contained wild-type bacteria in large spacious phagosomes (*A* and *B*). These bacteria were in membrane-bound vacuoles (*B*, black arrows) as well as in vacuoles in which the membrane had been either partially or fully degraded (*A* and *B*, white arrows). Some wild-type bacteria were free in the cytoplasm and were not membrane bound (*C*, white arrow). In contrast, the *sar*− mutant 11D2 was in small vacuoles with closely apposed cell membranes (tight phagosomes) (*D*, black arrows). Magnification: *A*, ×10,000; *B*, ×8,000; C, ×25,000; *D*, ×17,000.

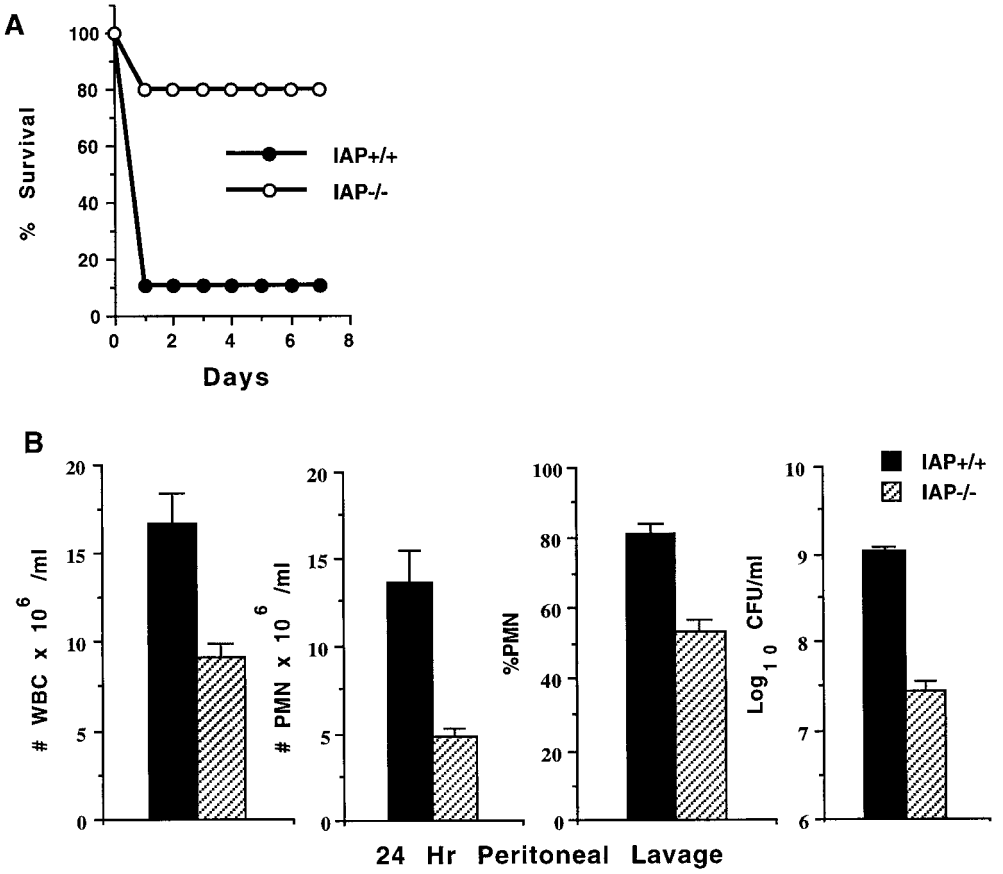

FIGURE 3. Response of $IAP^{+/+}$ and $IAP^{-/-}$ mice to lethal *S. aureus* infection. *A*, Effect of 1–2 × 10^6 SA1 given i.p. on the survival of $IAP^{+/+}$ (n = 9) (●) and $IAP^{-/-}$ (n = 10) (○) mice. $IAP^{-/-}$ mice have significantly enhanced survival in response to SA1 infection (p = 0.0055). *B*, Comparison of the number of WBC per ml, number of PMN per ml, percentage of PMN, and log CFU per ml in a 5-ml peritoneal lavage from $IAP^{+/+}$ (n = 5) (■) and $IAP^{-/-}$ (n = 7) (▨) mice performed 24 h after i.p. challenge with 1.2 × 10^6 CFU of SA1. The numbers of WBC per ml, PMN per ml, percentage of PMN, and the bacterial burden were all significantly lower in $IAP^{-/-}$ mice (p = 0.0021, 0.0045, 0.0044, and 0.0044, respectively).

whether the same benefit of IAP deficiency could be observed with a sublethal infection. We injected $IAP^{+/+}$ and $IAP^{-/-}$ mice with a dose of SA1 that all wild-type mice survive (5 × 10^5 CFU) and assessed the leukocyte response and bacterial burden at 48 h after i.p. challenge. $IAP^{-/-}$ mice, as compared with $IAP^{+/+}$ mice, had significantly fewer PMN (0.65 ± 0.4 × 10^5/ml vs 9.5 ± 1.4 × 10^5/ml, p = 0.0039) and a significantly reduced bacterial burden (0 CFU vs 3.75 ± 0.92 log CFU, p = 0.0021, n = 6) even in a sublethal infection (data not shown). These data suggest that limiting PMN migration even in response to a sublethal challenge of *S. aureus* results in the enhanced clearance of the pathogen from sites of infection.

Because IAP is expressed on all cells (27), the enhanced survival of the $IAP^{-/-}$ mice could be related to other consequences of IAP deficiency. To address this, we limited PMN influx by partially depleting PMN by injection of mAb anti-Gr-1 RB6-8C5 (22). The essential role of PMN in several murine models of infection, including *S. aureus*-induced arthritis (8, 22), has been demonstrated by total ablation of PMN with this Ab. In preliminary experiments, we found that i.v. administration of 5–10 μg of purified mAb RB6 vs an isotype control, SFR3, depleted 25–33% of bone marrow PMN, which resulted in a 50–66% reduction in the number of PMN migrating into the peritoneum in response to infection. A concentration of 100 μg depleted 90–100% of PMN migrating into the peritoneum. These single doses were sufficient to alter the number of PMN for 72 h.

To confirm the results of others that PMN play an essential protective role in *S. aureus* infection (8), we assessed the effect of complete ablation of PMN on *S. aureus*-induced peritonitis/sepsis. Wild-type mice were treated with 100 μg mAb RB6 to fully deplete the PMN migrating into the peritoneum 24 h before a sublethal dose of SA1. Greater than 90% depletion of the PMN resulted in a significant increase in the number of bacteria as compared with an Ab control (7.58 ± 0.05 log CFU vs 4.99 ± 0.15 log CFU, p = 0.002, n = 4) (data not shown). The RB6-treated mice were moribund in appearance, confirming that total PMN ablation is able to convert a sublethal infection to a lethal infection. These data indicate that PMN do play an essential protective role in *S. aureus* infection.

To test the effect of partial PMN depletion on survival against a lethal dose of *S. aureus*, we treated wild-type mice with 10 μg mAb RB6 or isotype control 24 h before i.p. challenge with 2 × 10^6 CFU of SA1. By 48 h, all of the control mice were moribund and succumbed to the infection. In contrast, the mAb RB6-treated mice all survived and appeared normal after 2–3 days (Fig. 4*A*, p = 0.004). To confirm that the enhanced survival of the mAb RB6-treated mice was due to a reduced bacterial burden and that this concentration of Ab only partially depleted PMN in the peritoneum, we assessed the number of PMN per ml and the CFU per ml in a lavage from the site of infection. Both of these parameters were significantly reduced in the mAb RB6-treated mice (Fig. 4*B*, hatched bars). Therefore, enhanced survival of the mAb RB6-treated mice coincided with an ~66% decrease in PMN number and with a ~2000-fold reduction in the number of bacteria at the site of infection. To confirm that this benefit could be observed with other *S. aureus* strains and that it was dose responsive, we

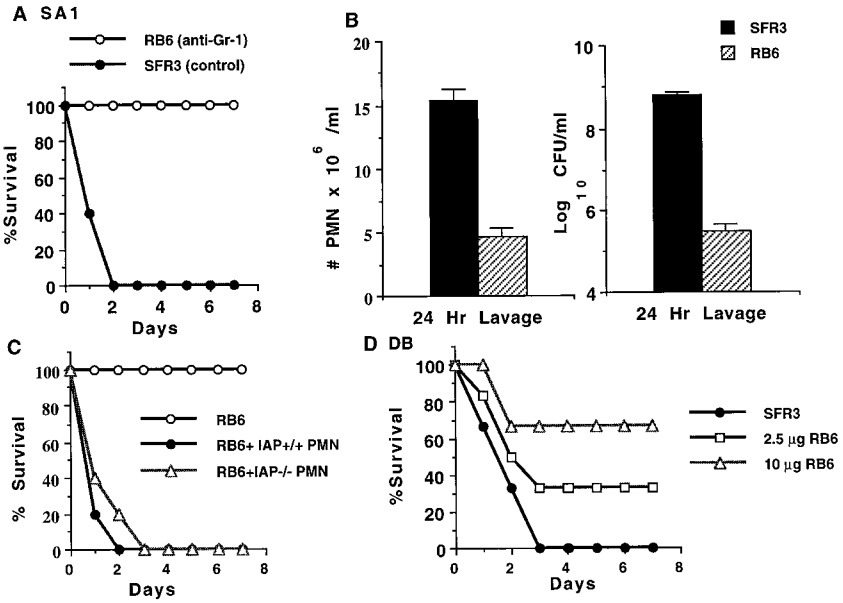

FIGURE 4. Effect of partial depletion of PMN with mAb RB6 as compared with isotype control SFR3 on the response to a lethal *S. aureus* infection in wild-type mice. *A*, Wild-type mice were given either 10 μg mAb RB6 ($n = 5$) or control SFR3 ($n = 5$) i.v. 24 h before i.p. challenge with 2×10^6 CFU SA1 and the percentage of survival was assessed over 7 days. RB6-treated mice have significantly enhanced survival in response to a lethal SA1 infection ($p = 0.004$, Fisher's exact test). *B*, The number of PMN/ml and log CFU/ml in a peritoneal lavage performed 24 h after i.p. challenge with 2×10^6 CFU of SA1 in mice treated with 10 μg of either mAb RB6 ($n = 4$) or control SFR3 ($n = 4$). The numbers of PMN per ml and the bacterial burden are significantly lower in RB6-treated mice ($p = 0.005$ and 0.0021, respectively). *C*, Effect of adding back PMN purified from either $IAP^{+/+}$ ($n = 5$) or $IAP^{-/-}$ ($n = 5$) mice vs saline ($n = 5$) on the survival of mAb RB6-treated wild-type mice in response to i.p. infection with 2×10^6 CFU of SA1. Wild-type mice were given 10 μg mAb RB6 i.v. 24 h before i.p. challenge with SA1. At the same time of the bacterial challenge, the mice were given i.v. 200 μl saline or 1×10^7 bone marrow PMN purified from either $IAP^{+/+}$ or $IAP^{-/-}$ mice. Both $IAP^{+/+}$ and $IAP^{-/-}$ PMN reverse the protection provided by partial depletion of PMN with mAb RB6 ($p = 0.004$). *D*, Dose-response effect of mAb RB6 administration on survival of mice injected i.p. with 1×10^9 DB. Survival was assessed in mice treated with 10 μg control SFR3 ($n = 6$), 2.5 μg mAb RB6 ($n = 6$), or 10 μg of mAb RB6 ($n = 6$) i.v. 24 h before i.p. challenge. mAb RB6 had a dose-responsive effect on the survival of mice infected with the microencapsulated strain, DB. Survival after treatment with 10 μg mAb RB6 was significantly enhanced as compared with mice treated with SFR3 ($p = 0.03$).

assessed the effect of two doses of RB6 on peritonitis induced by the microencapsulated strain DB. As shown in Fig. 4*D*, there was a dose-dependent effect of RB6 on DB-induced mortality ($p = 0.03$). In addition, we assessed whether RB6 treatment affected gentamicin-resistant, PMN-associated CFU. The RB6-treated mice had significantly fewer gentamicin-resistant, PMN-associated DB present at the site of infection than did the mice treated with the control SFR3 (2.84 ± 0.4 vs 5.89 ± 0.2 log CFU, $n = 4$). These data demonstrate that partial depletion of PMN significantly enhances host defense against *S. aureus*-induced peritonitis and sepsis and that this benefit arises at least in part by reducing the number of viable organisms inside PMN.

The above data indicated that limiting PMN migration into a site of *S. aureus* infection either by depletion with a mAb or by IAP deficiency could enhance host defense. These data implicate PMN in the pathogenesis of *S. aureus* infection. To show cause and effect, we asked whether repletion of PMN would reverse the beneficial effects of limiting PMN migration. Therefore, we treated wild-type mice with mAb RB6 to partially deplete PMN and administered i.v. either wild-type or $IAP^{-/-}$ PMN purified from bone marrow at the time of i.p. challenge with a lethal dose of SA1 and assessed survival. The survival at 48 h of RB6-treated mice dropped from 100% to 0% with the infusion of wild-type PMN and dropped from 100% to 20% with infusion of $IAP^{-/-}$ PMN (Fig. 4*C*, $p = 0.004$). These data indicate that the protection conferred by partial PMN depletion can be reversed by administration of purified PMN from either wild-type or $IAP^{-/-}$ mice. The ability of $IAP^{-/-}$ PMN to reverse this protection is not unexpected because $IAP^{-/-}$ PMN are not completely devoid of the ability to migrate into the peritoneum (Fig. 3*B* (19)), and their migration is greatly facilitated in $IAP^{+/+}$ mice (H. Gresham and F. Lindberg, unpublished observations). To confirm that the decreased survival with the infusion of PMN resulted in increased PMN migration into the peritoneum and an increased bacterial burden, we performed a lavage 24 h after SA1 challenge of RB6-treated mice in the presence and absence of additional wild-type or $IAP^{-/-}$ PMN. The number of both types of PMN was significantly increased as was the bacterial burden and both were similar to the values in SFR3-treated mice depicted in Fig. 4*B* (data not shown). Taken together, these data indicate that the protection provided by low dose mAb RB6 and IAP deficiency against *S. aureus*-induced peritonitis and sepsis is specifically mediated by their effects on PMN.

A trivial explanation for these data could be that IAP deficiency or mAb RB6 stimulates bactericidal killing by murine PMN, thus enhancing host defense. To address this, we assessed killing of DB by $IAP^{+/+}$, $IAP^{-/-}$, and RB6-treated wild-type PMN in vitro. Incubation of 2×10^6 DB with 1×10^6 of the various PMN for 2 h did not significantly reduce the log CFU (SFR3 CFU = 6.43, RB6 = 6.40, $IAP^{+/+}$ = 6.48, and $IAP^{-/-}$ = 6.51, data not shown). These data confirm those of others that indicate that in vitro killing of *S. aureus* by PMN is not optimal unless Ab and other opsonins are present (29).

Exogenous administration of the CXC chemokine, MIP-2, decreases survival and increases the bacterial burden in mice infected with S. aureus

Because the above data indicated that limiting PMN migration into the site of a *S. aureus* infection could be beneficial, we asked

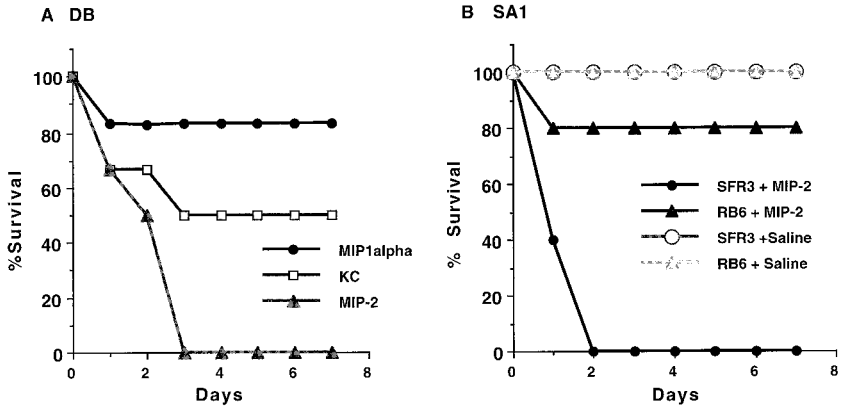

FIGURE 5. *A*, Effect of CXC chemokines as compared with a CC chemokine on the survival of mice infected with 3×10^8 DB. Mice ($n = 6$ for each group) were infected i.p. with DB and 4 h later given 400 ng of MIP-1-α, KC, or MIP-2. Treatment with the CXC chemokines, MIP-2 and KC, significantly decreased survival as compared with treatment with the CC chemokine MIP-1-α ($p = 0.0076$). *B*, Effect of partial PMN depletion on MIP-2-induced lethal *S. aureus* infection. Wild-type mice were given 10 μg of either mAb RB6 (△, ▲) or control SFR3 (○, ●) i.v. 24 h before i.p. challenge with 2×10^5 CFU of SA1 (a sublethal dose). Four hours after injection of the pathogen, the mice were given either saline (○, △) or 400 ng of MIP-2 (●, ▲). Percentage of survival was assessed at 7 days. Mice treated with control Ab and MIP-2 had significantly reduced survival as compared with saline controls ($p = 0.004$, $n = 5$ for each group). Moreover, treatment with mAb RB6 significantly reversed MIP-2-induced lethality ($p = 0.024$, $n = 5$ for each group).

whether promoting PMN migration would be deleterious. The murine CXC chemokine, MIP-2, is a potent chemotactic factor for murine neutrophils via the CXCR2 receptor (30). Therefore, we asked whether exogenous addition of MIP-2 or another murine CXC chemokine, KC, during a sublethal infection would be sufficient to diminish host defense against this pathogen. We challenged wild-type mice with a sublethal dose of DB and 4 h later injected i.p. MIP-2, KC, or the CC chemokine MIP-1α as a control. Both MIP-2 and KC significantly reduced survival of the mice as compared with the CC chemokine control (Fig. 5*A*, $p = 0.0076$). In addition, the timing of the MIP-2 administration was important. The maximal effect was observed at 4 h after the initiation of infection with minimal or no effect when given at the time of infection. To prove that this effect was mediated by PMN, we injected mice partially depleted of PMN by mAb RB6 vs SFR3 control with either saline or MIP-2 i.p. 4 h after challenge with a sublethal dose of SA1 and assessed survival. Mice treated with control mAb and saline all survived the infection, whereas mice treated with control mAb and MIP-2 all succumbed to the infection by day 2 (Fig. 5*B*, $p = 0.004$). In contrast, 80% of the mice that had been partially depleted of PMN with mAb RB6 before treatment with MIP-2 survived this infection ($p = 0.024$). Partial PMN depletion had no effect on the survival of saline-injected mice. These data indicate that CXC chemokine administration is sufficient to convert a sublethal *S. aureus* infection into a lethal infection and that PMN are primarily responsible for this deleterious effect.

To confirm that the increased lethality of MIP-2 was due to an increase in bacterial burden, we assessed the effect of MIP-2 on the number of PMN per ml and the log CFU in peritoneal lavages 24 h after i.p. challenge with a sublethal dose of DB, the *sar*− mutant 11D2, SA1, and RN6390. As shown in Fig. 6, MIP-2 significantly increased the percentage and number of PMN present in the peritoneum in response to infection with a sublethal challenge of both DB and 11D2. However, the bacterial burden was significantly increased only with DB infection ($p = 0.006$) and not with the *sar*− mutant 11D2. Qualitatively identical data were obtained with SA1 and with RN6390 infection as with DB (data not shown). Moreover, the number of gentamicin-resistant, PMN-associated CFU in the presence of MIP-2 was greater for DB than for 11D2 (4.93 ± 0.42 log CFU vs 1.67 ± 0.11, $n = 3$). The number of PMN-associated, gentamicin-resistant PMN was <1 log CFU for both strains at this sublethal dose in the absence of exogenous MIP-2 administration (data not shown). In addition, MIP-2 administration enhanced infection in $IAP^{-/-}$ mice (7.93 ± 0.44 log CFU vs saline control 5.37 ± 0.37 log CFU, $n = 3$), indicating that the benefit of IAP deficiency could be reversed by increased PMN migration (data not shown). These data indicate that pathogenic strains of *S. aureus* demonstrate enhanced survival in the presence of increased numbers of PMN, both wild type and IAP deficient, and that the ability to do this is regulated by *sar*.

S. aureus *taken up in vitro by CXC chemokine-stimulated PMN remain viable intracellularly*

The above data indicated that CXC chemokine administration increased the number of viable *S. aureus* inside PMN. This raised the

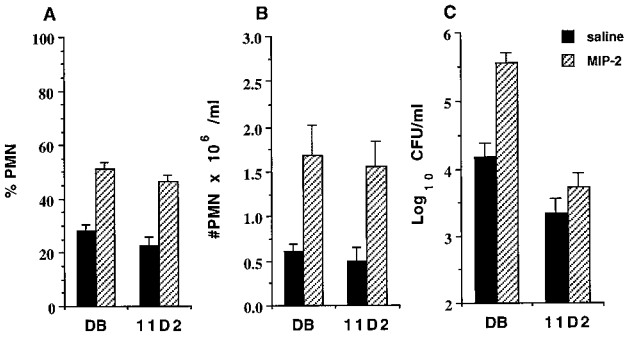

FIGURE 6. Effect of MIP-2 on sublethal *S. aureus* infection with bacterial strains DB (*sar*+) and 11D2 (*sar*−). Wild-type mice were injected i.p. with 1×10^7 CFU of either DB or 11D2. After 4 h, the mice were injected i.p. with either sterile, endotoxin-free saline ($n = 4$–6 mice) or 400 ng MIP-2 ($n = 4$–7 mice). Twenty-four hours after i.p. challenge, a peritoneal lavage was performed and the percentage of PMN, number of PMN per ml, and the $\log_{10}$ CFU per ml were assessed. MIP-2 administration significantly increased all of these parameters for DB ($p = 0.0029$, 0.038, and 0.006, respectively). For 11D2, only the percentage of PMN and the number of PMN per ml, and not the log CFU per ml, were significantly increased with MIP-2 ($p = 0.0033$ and 0.015, respectively). The *sar*− mutant, which lacks the expression of several virulence factors, is less able to survive and avoid clearance in the presence of excess PMN as compared with wild-type *S. aureus*.

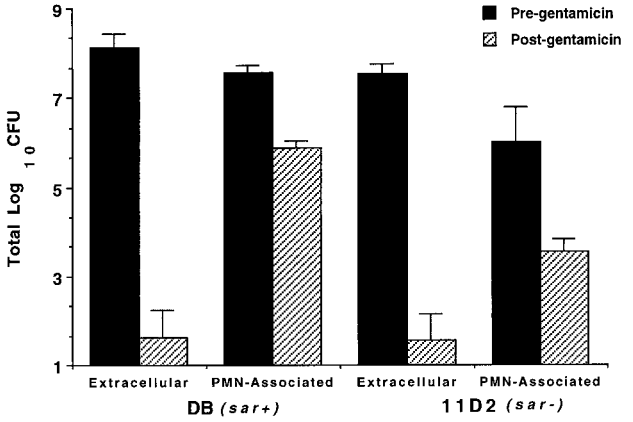

FIGURE 7. In vitro uptake and intracellular survival of *S. aureus* incubated with PMN and IL-8. Human PMN (1×10^7) were incubated with 10^8 CFU of either DB or the *sar*− strain 11D2 in the presence of 500 ng human IL-8. After 4 h at 37°C, the extracellular CFU in the supernatant was separated from the PMN by centrifugation. The number of extracellular CFU and the number of PMN-associated CFU were determined by serial dilution and spread-plating before and after incubation with 100 μg/ml gentamicin for 2 h at 37°C. The extracellular bacteria were susceptible to killing by gentamicin. However, strain DB (*sar*+) had significantly more PMN-associated gentamicin-resistant CFU than did strain 11D2 (*sar*−).

question of whether simply increasing the number of PMN was sufficient or whether the chemokines played an additional role in enhancing uptake of the bacteria. To address this, we developed an in vitro assay with human PMN that would duplicate what we observed in vivo in the mice. Human PMN were incubated with a 10:1 ratio of *S. aureus* (strains DB, *sar*− 11D2, and SA1) in the presence of the human CXC chemokine, IL-8, to mimic the relative numbers of PMN, bacteria, and MIP-2 that we observed in the mice. After 4 h, the extracellular supernatant was separated from the PMN, and both were subjected to gentamicin treatment. The extracellular CFU of both DB and 11D2 were killed by gentamicin (Fig. 7). In contrast, the number of viable CFUs associated with PMN after gentamicin treatment were significantly higher for DB than for 11D2. Qualitatively identical data were obtained with SA1 as with DB (data not shown). Minimal intracellular survival of the bacteria was observed in buffer-treated, CC chemokine-stimulated, or phorbol ester-stimulated PMN (data not shown). MIP-2 stimulation of murine PMN also promoted uptake and intracellular survival of DB in vitro (data not shown). To assess how blood-derived opsonins may affect these results, we also included 10% fresh autologous human serum containing agglutinating Ab and measured the number of gentamicin-resistant, PMN-associated DB. In the presence of IL-8 alone, the log CFU of the gentamicin-resistant, PMN-associated DB was 6.63 ± 0.21, $n = 3$. With the addition of 10% serum the number was significantly reduced to 5.80 ± 0.014, $n = 3$. However, the addition of serum-derived opsonins did not ablate the ability of DB to remain viable inside PMN, reflecting what we observed in vivo (Fig. 1*A*). These data suggest that CXC chemokines both promote PMN migration and uptake of *S. aureus* into a compartment that permits its survival. Moreover, these data indicate that IL-8-stimulated human PMN, as well as MIP-2-stimulated murine PMN, provide a sufficient environment for the uptake and intracellular survival of *S. aureus* and that intracellular survival is attenuated in a mutant *sar*− strain.

Discussion

This work provides both in vivo and in vitro evidence that *S. aureus* can survive inside PMN and that this ability is regulated, at least in part, by the global regulator, *sar*, which governs the synthesis and secretion of several virulence factors (2). These data extend observations made in vitro with PMN and macrophages in the 1950s and 1960s (11–14) and support current in vitro studies of *S. aureus* invasion and survival in epithelial cells, endothelial cells, and osteoblasts (16–18). Taken together, these data clearly indicate that the pathogenesis of *S. aureus* infection involves both extracellular and intracellular locales. Moreover, our data suggest that *S. aureus*, like bona fide intracellular pathogens (26), has the ability to invade and survive inside the very cell that is responsible for its destruction. Our electron microscopy studies and those assessing invasion of epithelial cells (17) suggest a possible mechanism by which this could occur. In both epithelial cells and PMN, wild-type bacteria are taken up into large spacious vacuoles resembling macropinosomes which arise from the formation of membrane ruffles that fold back onto the surface of the cell (24). These vacuoles are identical in appearance with those observed in both epithelial cells and macrophages invaded by *Salmonella* and which contribute to its intracellular survival (24–26). However, in PMN, wild-type bacteria are also taken up into smaller vacuoles with closely apposed cell membranes that are identical with those that arise from repeated receptor-ligand interactions that drive pseudopod advance over the surface of the pathogen (the zipper mechanism) (25). Because uptake of the *sar*− mutant, which is attenuated in its ability to survive inside PMN, is primarily into these tight phagosomes, these smaller vacuoles may represent phagosomes that are competent for fusion with azurophil granules (lysosomal compartment in PMN) and thus represent a site of intracellular destruction. In contrast, the macropinosome membrane may not be competent for fusion with azurophil granules, thus allowing sufficient time for lysis of the vacuolar membrane and escape of the bacteria into the cytoplasm. Once free in the cytoplasm, the pathogen may replicate (17). In this paradigm, PMN possess two intracellular locales, one that contributes to survival (macropinosome) and another to the destruction (phagolysosome) of the pathogen. In this manner, PMN could play both an essential protective role and an inadvertent deleterious role.

Whereas *S. aureus* invasion of both epithelial cells and PMN results in uptake into large vacuoles, our data with PMN differ from those with epithelial cells in that an additional exogenous factor is required. We found that incubation of PMN with the CXC chemokines, IL-8, MIP-2, or KC, was required for optimal uptake and intracellular survival. This is perhaps not surprising because chemotactic factors promote membrane ruffling of PMN (31). In contrast, neither the CC chemokine, MIP-1-a, which has minimal effects on PMN function (30), nor phorbol esters, which stimulate many PMN functions, were able to promote uptake and survival of *S. aureus*. Because exogenous administration of CXC chemokines in vivo enhanced the bacterial burden and the number of viable bacteria associated with PMN, production of these chemokines in vivo may contribute significantly to the pathogenesis of *S. aureus* infection. Preliminary studies indicate that levels of MIP-2 and KC in vivo are ~1000-fold higher with wild-type *S. aureus* infection than with the *sar*− mutant (H. D. Gresham, unpublished observation). Therefore, *sar* may regulate invasion and intracellular survival in PMN by affecting both the synthesis of CXC chemokines and uptake by the PMN. In this regard, recent evidence indicates that fibronectin binding protein, the expression of which is regulated by *sar* (2, 3), is required for efficient invasion of epithelial cells (32). Studies are ongoing in our laboratories to assess whether fibronectin-binding protein regulates both CXC chemokine synthesis and intracellular survival in PMN in vivo.

Because we were interested in the consequences of the survival of *S. aureus* inside PMN as compared with other cell types, we asked whether limiting access to this privileged site would be beneficial to host defense. Although not particularly feasible with endothelial or epithelial cells, it is possible to test this by controlling the number of PMN at the site of infection. To achieve this, we used IAP-deficient mice and mice treated with low doses of mAb RB6 to limit the number of PMN at the site of infection. Both of these experimental approaches benefited host defense and reduced the bacterial burden. We believe that this enhanced host defense was provided by affecting PMN specifically because the benefit of both IAP deficiency and partial PMN depletion with mAb RB6 could be reversed by giving additional PMN and by promoting PMN migration with MIP-2 and KC administration. Although these experimental approaches reduced the number of viable bacteria, the precise mechanism for this remains unclear. The possibilities for this benefit include: 1) enhanced bactericidal killing by $IAP^{-/-}$ and RB6-treated PMN; 2) elimination of an intracellular reservoir of replicating organisms; 3) reduced CXC chemokine production and thus macropinocytic uptake; and 4) failure to sequester organisms away from the cells or agents that are competent for killing. We found that $IAP^{-/-}$ and RB6-treated PMN did not exhibit enhanced bactericidal activity in vitro. Moreover, we currently have no evidence to support replication of the organism inside PMN even though it may occur in other cells (17). Therefore, we believe that some combination of possibilities 3 and 4 are most likely to be operative. For example, reducing CXC chemokine synthesis which is essential for intracellular survival could facilitate opsonin-dependent uptake into tight phagosomes and thus result in destruction of the pathogen. In addition, reducing the number of PMN and thereby the number of intracellular organisms could simply increase the number of extracellular organisms accessible to extracellular bactericidal agents such as antimicrobial polypeptides (33) and type II phospholipase A_2 (34). Further experimentation will be required to discriminate between these possibilities.

These experimental data raise the issue of whether survival of *S. aureus* inside PMN occurs during a natural infection. To begin to address this, we obtained fluid from an abscess that developed at the site of a surgical incision and that was shown subsequently to be infected with *S. aureus*. In this single very preliminary experiment, we found that PMN isolated from this site contained 5.5×10^6 CFU/1.0×10^7 PMN. Moreover, after incubation of the PMN with gentamicin, the CFU remained high at 4.3×10^6/ml (H. Gresham, unpublished observation). These data suggest that at sites of *S. aureus*-induced abscess formation, which occurs in the skin and in deep organs such as the liver, the appropriate environmental conditions may exist that would favor persistence of the organism by this mechanism. From our data, we hypothesize that this would minimally include: 1) expression by the bacteria of virulence determinants regulated by *sar*; 2) large numbers of viable PMN; 3) stimulated secretion of nanogram quantities of CXC chemokines; and 4) attenuated access of blood-derived opsonins to the site of infection. These conditions would favor uptake of the bacteria into macropinosomes as compared with tight phagosomes and thus perpetuate their survival even though PMN are present. Further experimentation will be required to determine whether this occurs during the course of natural infection at other sites.

Our data demonstrate that *S. aureus* has the ability to exploit the inflammatory response of the host to facilitate its own survival by gaining access to a locale within PMN where it is prevented from being killed. Moreover, the global regulator, *sar*, modulates this ability, indicating that it is a virulence determinant for this pathogen. In addition, our data suggest that successful therapeutic strategies for treating this infection could arise by manipulating the response of the host to block uptake of the pathogen into macropinosomes and/or to promote uptake into phagolysosomes for destruction.

Acknowledgments

We thank Cheryl Pulaski for technical assistance in the completion of these studies and Jan Pfeiffer for assistance with the electron microscopy studies.

References

1. Lowy, F. D. 1998. *Staphylococcus aureus* infections. *N. Engl. J. Med. 339:520.*
2. Cheung, A., J. M. Koomey, C. Butler, S. Projan, and Fischetti, V. 1992. Regulation of exoprotein expression in *Staphylococcus aureus* by a locus (*sar*) distinct from *agr*. *Proc. Natl. Acad. Sci. USA 89:6462.*
3. Cheung, A., M. Yeaman, P. Sullam, M. Witt, and Bayer, A. 1994. Role of the *sar* locus of *Staphylococcus aureus* in induction of endocarditis in rabbits. *Infect. Immun. 62:1719.*
4. Nilsson, I.-M., J. Patti, T. Bremell, M. Hook, and Tarkowski, A. 1998. Vaccination with a recombinant fragment of collagen adhesin provides protection against *Staphylococcus aureus*-mediated septic death. *J. Clin. Invest. 101:2640.*
5. Balaban, N., and Novick, R. 1995. Autocrine regulation of toxin synthesis by *Staphylococcus aureus. Proc. Natl. Acad. Sci. USA 92:1619.*
6. Balaban, N., T. Goldkorn, R. Nhan, L. Dang, S. Scott, R. Ridgley, A. Rasooly, S. Wright, J. Larrick, R. Rasooly, et al. 1998. Autoinducer of virulence as a target for vaccine and therapy against *Staphylococcus aureus. Science 280:438.*
7. Cheung, A., K. Eberhardt, E. Chung, M. Yeaman, P. Sullam, M. Ramos, and A. Bayer. 1994. Diminished virulence of a *sar*−/*agr*− mutant of *Staphylococcus aureus* in the rabbit model of endocarditis. *J. Clin. Invest. 94:1815.*
8. Verdrengh, M., and A. Tarkowski. 1997. Role of neutrophils in experimental septicemia and septic arthritis induced by *Staphylococcus aureus. Infect. Immun. 65:2517.*
9. Lowrance, J., F. O'Sullivan, T. Caver, W. Waegell, and H. Gresham. 1994. Spontaneous elaboration of transforming growth factor-β suppresses host defense against bacterial infection in autoimmune MRL/lpr mice. *J. Exp. Med. 180:1693.*
10. Caver, T. E., F. O'Sullivan, L. Gold, and H. Gresham. 1996. Intracellular demonstration of active TGFβ1 in B cells and plasma cells of autoimmune mice: IgG-bound TGFβ1 suppresses neutrophil function and host defense against *Staphylococcus aureus* infection. *J. Clin. Invest. 98:2496.*
11. Rogers, D., and M. Melly. 1965. Speculation on the immunology of staphylococcal infections. *Ann. NY Acad. Sci. 128:274.*
12. Rogers, D., and R. Tompsett. 1952. The survival of staphylococci within human leukocytes. *J. Exp. Med. 95:209.*
13. Melly, M., J. Thomison, and D. Rogers. 1960. Fate of staphylococci within human leukocytes. *J. Exp. Med. 112:1121.*
14. Kapral, F., and F. Shayegani. 1959. Intracellular survival of staphylococci. *J. Exp. Med. 110:123.*
15. Craven, N., and Anderson, J. 1979. The location of *Staphylococcus aureus* in experimental chronic mastitis in the mouse and the effect on the action of sodium cloxacillin. *Br. J. Exp. Pathol. 60:453.*
16. Vesga, O., M. Groeschel, M. Otten, D. Brar, J. Vann, and R. Proctor. 1996. *Staphylococcus aureus* small colony variants are induced by the endothelial cell intracellular milieu. *J. Infect. Dis. 173:739.*
17. Bayles, K., C. Wesson, L. Liou, L. Fox, G. Bohach, and W. Trumble. 1998. Intracellular *Staphylococcus aureus* escapes the endosome and induces apoptosis in epithelial cells. *Infect. Immun. 66:336.*
18. Hudson, M., W. Ramp, N. Nicholson, A. Williams, and M. Nousiainen. 1995. Internalization of *Staphylococcus aureus* by cultured osteoblasts. *Microb. Pathog. 19:409.*
19. Lindberg, F., D. Bullard, T. Caver, H. Gresham, A. Beaudet, and E. Brown . 1996. Decreased resistance to bacterial infection and granulocyte defects in IAP-deficient mice. *Science 274:795.*
20. Lee, J. C., M. J. Betley, C. A. Hopkins, N. E. Perez, and G. Pier. 1987. Virulence studies, in mice, of transposon-induced mutants of *Staphylococcus aureus* differing in capsule size. *J. Infect. Dis. 156:741.*
21. Pfeiffer, J., J. Seagrave, B. Davis, G. Deanin, and J. Oliver. 1985. Membrane and cytoskeletal changes associated with IgE-mediated serotonin release from rat basophilic leukemia cells. *J. Cell Biol. 101:2145.*
22. Vassiloyanakopoulos, A., S. Okamoto, and Fierer, J. 1998. The crucial role of polymorphonuclear leukocytes in resistance to *Salmonella dublin* infections in genetically susceptible and resistant mice. *Proc. Natl. Acad. Sci. USA 95:7676.*
23. Gresham, H. D., C. J. Ray, and F. O'Sullivan. 1991. Defective neutrophil function in the autoimmune mouse strain MRL/lpr: potential role of transforming growth factor-β. *J. Immunol. 146:3911.*

24. Swanson, J. A., and C. Watts. 1995. Macropinocytosis. *Trends Cell Biol. 5:424.*
25. Swanson, J. A., and S. Baer. 1995. Phagocytosis by zippers and triggers. *Trends Cell Biol. 5:89.*
26. Finlay, B. B., and S. Falkow. 1997. Common themes in microbial pathogenicity revisited. *Microbiol. Mol. Biol. Rev. 61:136.*
27. Lindberg, F., H. Gresham, E. Schwarz, and E. Brown. 1993. Molecular cloning of integrin-associated protein: an immunoglobulin family member with multiple membrane spanning domains implicated in $\alpha_V\beta_3$-dependent ligand binding. *J. Cell Biol. 123:485.*
28. Cooper, D., F. Lindberg, J. Gamble, E. Brown, and M. Vadas. 1995. Transendothelial migration of neutrophils involves integrin-associated protein (CD47). *Proc. Natl. Acad. Sci. USA 92:3978.*
29. Xu, S., R. D. Arbeit, and J. C. Lee. 1992. Phagocytic killing of encapsulated and microencapsulated *Staphylococcus aureus* by human polymorphonuclear leukocytes. *Infect. Immun. 60:1358.*
30. Rollins, B. J. 1997. Chemokines. *Blood 90:909.*
31. Anderson, D., F. Schmalstieg, M. Arnaout, S. Kohl, M. Tosi, N. Dana, C. Buffone, B. Hughes, B. Brinkcey, W. Dickey, et al. 1984. Abnormalities of polymorphonuclear leukocyte function associated with a heritable deficiency of high molecular weight surface glycoproteins: common relationship to diminished cell adherence. *J. Clin. Invest. 74: 536.*
32. Dziewanowska, K., J. Patti, C. Deobald, K. Bayles, W. Trumble, and G. Bohach. 1999. Fibronectin binding protein and host cell tyrosine kinase are required for internalization of *Staphylococcus aureus* by epithelial cells. *Infect. Immun. 67: 4673.*
33. Dhawanm V., M. Yeamn, A. Cheung, E. Kim, P. Sullam, and A. Bayer. 1997. Phenotypic resistance to thrombin-induced platelet microbicidal protein in vitro is correlated with enhanced virulence in experimental endocarditis due to *S. aureus. Infect. Immun. 65:3293.*
34. Dominiecki, M., and J. Weiss. 1999. Antibacterial action of extracellular mammalian group IIA phospholipase A2 against grossly clumped *Staphylococcus aureus. Infect. Immun. 67:2299.*