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# Role of Coagulase in a Murine Model of Hematogenous Pulmonary Infection Induced by Intravenous Injection of *Staphylococcus aureus* Enmeshed in Agar Beads

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**We describe a novel mouse model of acute staphylococcal pneumonia induced by intravenous injection of *Staphylococcus aureus* enmeshed in agar beads. For comparison, we also used various strains of bacteria, including three strains of *S. aureus*, two strains of *Staphylococcus epidermidis*, one strain of *Streptococcus pyogenes*, three strains of *Pseudomonas aeruginosa*, and one strain of *Klebsiella pneumoniae*. All except two strains of *S. aureus* were cleared rapidly from the lungs. When *S. aureus* NUMR1 enmeshed in agar beads was injected intravenously, the organisms concentrated and remained in the lung for a period longer than several weeks. Multiple lung abscesses were evident macroscopically, and histological examination of the infected lung showed multiple lung abscesses around the pulmonary arterioles, consisting of bacterial colonies encircled with fibrin filaments and surrounded by inflammatory cells of neutrophils and macrophages. When 14 strains of clinically isolated *S. aureus* were injected intravenously, the number of bacteria recovered from the lung tissue 7 days after infection correlated with the titer of staphylocoagulase ($P < 0.01$) but not with the titer of clumping factor. Injection of coagulase-deficient mutant strain DU5843 was associated with a markedly reduced number of viable bacteria isolated from the lung, compared with its coagulase-positive parental strain DU5789. Our results suggest that coagulase may play a role in the development of blood-borne staphylococcal pneumonia in our model. Our animal model is simple and reproducible and resembles blood-borne staphylococcal pneumonia in humans, and it could be useful for investigating the pathogenicity or treatment of staphylococcal pulmonary infection, including infections with methicillin-resistant *S. aureus*.**

Laboratory models of staphylococcal murine pulmonary infection have been used previously. In most instances, the bacteria are instilled into the trachea (7, 8) or administered by aerosol (5, 9–11, 24, 25). When the organisms are instilled intratracheally, they are eliminated rapidly by phagocytic ingestion (9, 10) and other extracellular killing mechanisms (11, 24). For example, DeMaria and Kapral (5) demonstrated that elimination of at least 99% of the inoculum occurs within 48 h in mice inoculated with $10^8$ CFU of staphylococci by this method. However, the lack of a satisfactory animal model, in which pathogens remain in the lung in adequate numbers for a sufficient period of time, manifesting clear pathologic features of infection, has been one of the major impediments to the study of the pathogenesis and therapy of staphylococcal pulmonary infection.

Staphylococcal infection develops in the lower respiratory tract as a result of bronchogenic or hematogenous spread. The hematogenous spread results from the release of staphylococci from an intravascular focus into the bloodstream. The organisms are then transported via the pulmonary circulation into one or more regions of the lung, where infection is established. Subjects at high risk for blood-borne staphylococcal pneumonia include intravenous drug abusers (18, 28), patients with infected vegetation of tricuspid valves (22), and patients with infection of the soft tissues (23).

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In the present study, to simulate human blood-borne staphylococcal pneumonia, we developed a new murine model of pulmonary infection of *Staphylococcus aureus* by intravenous injection of the bacteria enmeshed in agar beads. Using this model, we investigated the virulence of proliferation of the organisms in the lung. *S. aureus* produces many extracellular products which may act as virulence factors. However, the exact role of the potential virulence determinants in different types of infections remains unclear. Among these factors, staphylocoagulase has been considered one of the most reliable determinants for the identification and differentiation of this bacterial species from other staphylococci and has been considered an important virulence factor. However, although several studies have examined this enzyme, its role in staphylococcal infections is still not clear. Using a rat endocarditis model, Moreillon et al. (21) demonstrated that the staphylocoagulase did not affect the initiation of experimental endocarditis, but they did not rule out a role of coagulase in the later phase of infection. In our murine model, as the infected organisms are isolated constantly for more than several weeks, we can analyze the virulence factors of the late phase of infection. Using this model, we studied the pathogenic role of staphylocoagulase and clumping factor in intrapulmonary bacterial proliferation.

## MATERIALS AND METHODS

**Animals.** Male ddY, 6-week-old, specific-pathogen-free mice (body weight, 20 to 25 g) were purchased from Shizuoka Agricultural Cooperative Association Laboratory Animals (Shizuoka, Japan). All animals were housed in a pathogen-free environment and provided with sterilized food and water in the Laboratory Animal Center for Biomedical Science at Nagasaki University. The experimental

protocol was approved by the Animal Care Ethics Review Committee of our institution.

**Bacterial strains.** Seventeen strains of *S. aureus* were examined. Two of these strains were standard strains (ATCC 29213, 25923), while the others, including five strains of methicillin-resistant *S. aureus*, were isolated clinically at Nagasaki University Hospital from blood, sputum, and pus samples. We also used two strains of *S. epidermidis* (SE397, SE398) and two of *Pseudomonas aeruginosa* (B1, B2) isolated from blood samples at Nagasaki University and standard strains of *Streptococcus pyogenes* (ATCC 19615), *P. aeruginosa* (ATCC 27853), and *Klebsiella pneumoniae* (ATCC 29665). The coagulase-positive *S. aureus* strain DU5789 and its coagulase-negative mutant DU5843, kindly provided by T. J. Foster (Moyne University, Dublin, Ireland) (26), were used to examine the role of coagulase in the pathogenesis of blood-borne experimental staphylococcal pneumonia. Coagulase-negative mutant DU5843 had undergone site-specific mutagenesis. The bacteria were stored at −70°C in brain heart infusion (BHI) broth (BBL Microbiology System, Cockeysville, Md.) supplemented with 10% (vol/vol) glycerol (Wako Pure Chemical Industries, Osaka, Japan) and 5% (wt/vol) skim milk (Yukijirushi Co., Tokyo, Japan) until use.

**Inoculum.** We used the method of Cash et al. (3) with some modification to prepare agar beads. *S. aureus* was cultured on a Trypticase soy agar (TSA) (BBL)-based sheep blood agar plate for 24 h at 37°C. The bacteria were suspended in endotoxin-free sterile saline and harvested by centrifugation (3,000 × *g*, 4°C, 10 min). The organisms were resuspended in cold sterile saline and diluted to $2 \times 10^9$ to $4 \times 10^9$ CFU/ml, as estimated by turbidimetry. The suspension was warmed to 45°C, and then 10 ml of the suspension was mixed with 10 ml of 4% (wt/vol) molten Noble agar (Difco Laboratories, Detroit, Mich.) at 45°C. The agar-bacterium suspension (1.0 ml) was placed into a 1.0-ml syringe, and the suspension was injected quickly with a 26-gauge needle into 49 ml of rapidly stirring ice-cooled sterile saline. This resulted in solidification of the agar droplets into beads of approximately 200 μm in diameter. The final concentration of agar was 0.04% (wt/vol), and the final number of bacteria was $2 \times 10^7$ to $4 \times 10^7$ CFU/ml. The 50% lethal dose ($LD_{50}$) of sterile agar beads without the organism was 0.32% determined in preliminary studies, and the animals inoculated with 0.04% (0.125 $LD_{50}$) agar beads were all alive.

**Experimental model.** We injected 0.20 to 0.25 ml of agar beads per mouse (10 μl/g of body weight), containing the bacteria suspended in saline, in the tail vein of each of five mice. Before the bacteria were enmeshed in agar beads, we verified their number by inoculating duplicates of serial dilutions onto blood agar plates and counting the CFU after 48 h of incubation at 37°C.

**Bacteriological and histopathological examination.** Each group of animals was sacrificed at specific time intervals by cervical dislocation. After exsanguination, the lung, liver, and kidney were dissected and removed under aseptic conditions. The organs used for bacteriological analyses were homogenized and cultured quantitatively by serial dilutions on blood agar plates. The lung tissue used for histological examination was fixed in 10% buffered formalin and stained with hematoxylin-eosin. The tissue was also stained with phosphotungstic acid-hematoxylin to examine for the presence of fibrin.

**Assay of coagulase.** Coagulase was assayed by a modified method of Jordens et al. (16). Twofold dilutions of an overnight culture in BHI broth (BBL) were prepared in sterile BHI broth to yield 100-μl volumes. To this we added 0.5-ml amounts of rabbit plasma (fresh-frozen dry plasma; Eiken Chemical Co., Tokyo, Japan) diluted 1 to 20 by BHI broth. Tubes were incubated for 2 h at 37°C. The highest dilution giving a definite clot was considered the coagulase titer.

**Assay of clumping factor.** Clumping factor was assayed by the method of Espersen et al. (6) with some modification. Briefly, the titer of clumping factor of staphylococci was measured quantitatively by the slide test. Staphylococci were grown overnight in TSA (BBL)-based sheep blood agar, collected with a toothpick, and spotted onto a clean microscope slide. Serial twofold dilutions of a 0.2% (wt/vol) human fibrinogen (Sigma Chemical Co., St. Louis, Mo.) solution were prepared in PBS (0.053 M $Na_2HPO_4$, 0.013 M $KH_2PO_4$ [pH 7.4]), and 40 μl was added and immediately mixed. The reciprocal of the highest dilution of fibrinogen showing clumping after 3 min was recorded as the titer of the clumping factor.

**Statistical analysis.** The Student *t* test was used to compare paired data, and a *P* value less than 5% denoted statistical significance.

## RESULTS

**Bacterial clearance.** The numbers of various strains of bacteria recovered from each group of lungs 7 days after inoculation are shown in Table 1. Only two of three strains of *S. aureus* (NUMR1 and ATCC 29213) proliferated significantly in the lungs. All other strains and bacteria were cleared rapidly from the lung, although certain bacteria were still isolated in small numbers.

**Serial changes in viable *S. aureus* NUMR1.** The serial changes in the number of viable *S. aureus* NUMR1 in the lung, liver, and kidney in our murine model are shown in Table 2. Two types of inocula ($4 \times 10^6$ CFU/mouse) were used in this series of experiments. The first consisted of *S. aureus* enmeshed in agar beads while the other was *S. aureus* suspended in saline. Injection of each inoculum was performed to examine the bacterial clearance from each organ. The mean bacterial count in the lungs of mice injected with bacteria enmeshed in agar beads was significantly higher on days 1, 8, and 12 compared with those on the corresponding days in mice injected with bacteria suspended in saline. During the experiment, the number of viable organisms recovered from the lung for mice injected with bacteria enmeshed in agar beads was 1.5 to 3.0 log CFU/organ higher than the number in the liver and kidney.

TABLE 1. Number of viable bacteria recovered from the lung 7 days after inoculation

| Strain | Bacterial count ($log_{10}$ CFU) | |
|---|---|---|
| | Inoculated | Recovered |
| *Staphylococcus aureus* | | |
| NUMR1 | 6.54 | 5.52 ± 1.23[a] |
| ATCC 29213 | 6.43 | 8.00 ± 0.59 |
| ATCC 25923 | 6.73 | ND[b] |
| *Staphylococcus epidermidis* | | |
| SE397 | 6.45 | ND |
| SE398 | 6.36 | ND |
| *Streptococcus pyogenes* ATCC 19615 | 5.08 | ND |
| *Pseudomonas aeruginosa* | | |
| ATCC 27853 | 6.78 | 1.25 ± 0.56 |
| B1 | 5.56 | 1.55 ± 0.52 |
| B2 | 6.99 | ND |
| *Klebsiella pneumoniae* ATCC 29665 | 6.88 | 1.24 ± 0.39 |

[a] Values are means ± standard deviations for five mice in each set of experiments.
[b] ND, not detected.

**Histological examination.** The pathologic features of staphylococcal NUMR1 pneumonia were established by serial macroscopic and microscopic examinations. There was preferential abscess formation in the lung throughout the observation period compared with other organs; only very few macroscopic foci were detected in the kidneys. After 24 h of intravenous inoculation, a septic embolus of *S. aureus* enmeshed in agar beads was detected in the pulmonary artery with inflammatory cell accumulation in its wall. The embolus contained certain vehicles in which the bacteria proliferated. Four days after inoculation, an abscess with neutrophil aggregates, cell debris, and proliferating bacteria was observed in blood vessels. The perivascular tissue surrounding the blood vessel wall also showed accumulations of neutrophils and mononuclear cells. A large number of cells and bacteria filled the lumen of the bronchiole, probably originating from the nearby abscess. On day 10, multiple round nodular lesions were observed macroscopically in the peripheral regions of the lung (Fig. 1). Microscopic examination showed these lesions to be staphylococcal lung abscesses, each containing a central zone of a bacterial colony surround by neutrophils and macrophages (Fig. 2A).

Phosphotungstic acid-hematoxylin staining showed a linearly stained component on the periphery of the bacterial colony, separating *S. aureus* from accumulated neutrophils that were thought to be fibrin filaments (Fig. 2B). We also compared the pathologic features of lungs in animals injected only with agar beads with pathologic features of those injected with *S. aureus* in saline. No focal inflammatory infiltration was detected in the lungs in both groups. This was true in the early and late postinoculation periods.

TABLE 2. Comparison of the effect of the vehicle used for bacterial injection on bacterial count in organs

| Organ and injection method | No. of viable bacteria ($\log_{10}$ CFU)[a] at day postinfection | | | | |
|---|---|---|---|---|---|
| | 1 | 4 | 8 | 12 | 20 |
| Lung | | | | | |
| Agar beads | 5.28 ± 0.48[b] | 6.47 ± 1.53 | 5.52 ± 1.23[b] | 7.09 ± 0.44[c] | 4.31 ± 2.30 |
| Saline | 3.32 ± 0.71 | 4.82 ± 0.45 | 2.55 ± 1.42 | ND[d] | ND |
| Liver | | | | | |
| Agar beads | 3.80 ± 0.71 | 2.66 ± 0.42 | 2.75 ± 0.70 | 3.58 ± 2.44 | ND |
| Saline | 3.85 ± 0.09 | 3.80 ± 0.53 | 1.63 ± 0.59 | 1.23 ± 0.39 | ND |
| Kidney | | | | | |
| Agar beads | 2.38 ± 0.42 | 2.06 ± 0.26 | 1.51 ± 0.89 | 3.42 ± 2.25 | ND |
| Saline | 2.05 ± 0.98 | 3.26 ± 1.13 | 1.57 ± 0.50 | ND | ND |

[a] Values are means ± standard deviations for five mice in each set of experiments.
[b] $P < 0.05$ compared with saline.
[c] $P < 0.01$ compared with saline.
[d] ND, not detected.

**Recovery of viable bacteria from the lung.** The mean bacterial counts in the lungs of mice injected with 14 other strains of *S. aureus* enmeshed in agar beads were compared 3 and 7 days after inoculation. The number of viable bacteria recovered from the lung remained continuously high ($10^5$ to $10^7$ CFU/lung) following infection with 10 strains of *S. aureus*, but 2 strains declined to under $10^3$ CFU/g at day 7 and 2 strains had median numbers of bacteria recovered from the lungs (data not shown).

**Relationship between bacterial count in lung and coagulase titer.** As shown in Fig. 3A, a significant correlation was observed between coagulase titer and number of viable bacteria recovered from the lung on day 7 ($r = 0.78$, $P < 0.01$).

**Relationship between bacterial count in lung and clumping titer.** As shown in Fig. 3B, there was no correlation between the titer of clumping factor and number of viable bacteria recovered from the lung on postinoculation day 7.

**Recovery of coagulase-positive (DU5789) and -negative (DU5843) strains of *S. aureus* from the lung.** Qualitative cultures of the lungs of mice injected with coagulase-positive parental strain DU5789 and its coagulase-negative mutant strain of *S. aureus* enmeshed in agar beads were compared 7 days after inoculation. The number of CFU of strain DU5789 recovered from the lung was significantly higher than that of strain DU5843 recovered from infected lung ($P < 0.05$) (Table 3).

## DISCUSSION

Hematogenous pneumonia is usually due to the release of infected thrombotic material from the venous system or from infected tricuspid vegetations, with subsequent septic infarction. Musher and McKenzie (22) reported that pneumonia was due to airborne infection in 11 of 20 patients with pneumonia due to *S. aureus*, while in the other 9 patients, pneumonia was of hematogenous origin. Most of the latter cases in their study were patients with vascular prosthesis, hemodialysis, or intravenous drug therapy. Similarly, Naraqi and McDonnell (23) described 10 patients with *S. aureus* pneumonia caused by hematogenous dissemination from a focus of soft tissue infection.

There is no satisfactory animal model that allows the study of blood-borne pneumonia, since clearance of injected bacteria suspended in saline occurs rapidly in the lung. In addition, there is currently no method that produces a selective blood-borne pneumonia. We described in the present study a new animal model of a specific blood-borne pneumonia following intravenous injection of *S. aureus* enmeshed in agar beads. The use of agar beads to produce chronic bronchial infection was described originally by Cash et al. (3), who inoculated *P. aeruginosa*-enmeshed agar beads into the trachea of the rat. We modified and simplified this method by omitting the use of mineral oil and injecting agar beads enmeshed with bacteria directly into the murine tail vein. We used *S. aureus* since the pathogen is known to cause blood-borne pneumonia. We also used other bacteria in our preliminary experiments, such as *S. epidermidis*, *S. pyogenes*, *K. pneumoniae*, and *P. aeruginosa*. However, only *S. aureus* was found to proliferate in sufficient numbers in the lung. More importantly, these experiments demonstrated that only certain virulent strains of *S. aureus* were able to induce such proliferation.

Injection of the pathogen enmeshed in agar beads and the subsequent transport into pulmonary circulation probably resulted in entrapment of the 200-μm beads in the pulmonary microvasculature. Our results showed that the mean number of viable bacteria recovered from the lungs was 1.5 to 3.0 log CFU/g higher than that from the liver and kidney for more than 3 weeks. This was in contrast to the rapid clearance from

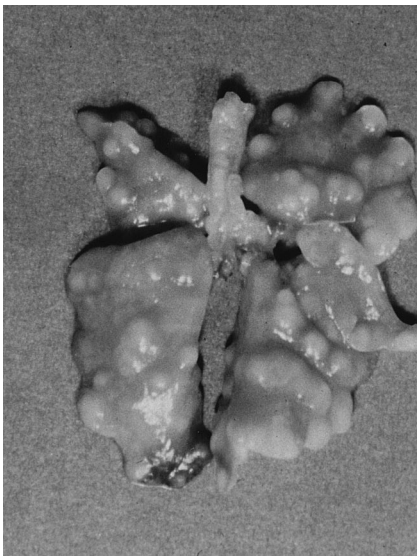

FIG. 1. Macroscopic features of the lungs infected with NUMR1 ten days after injection of *S. aureus* enmeshed agar beads. Note the multiple round abscesses in the periphery of the lung.

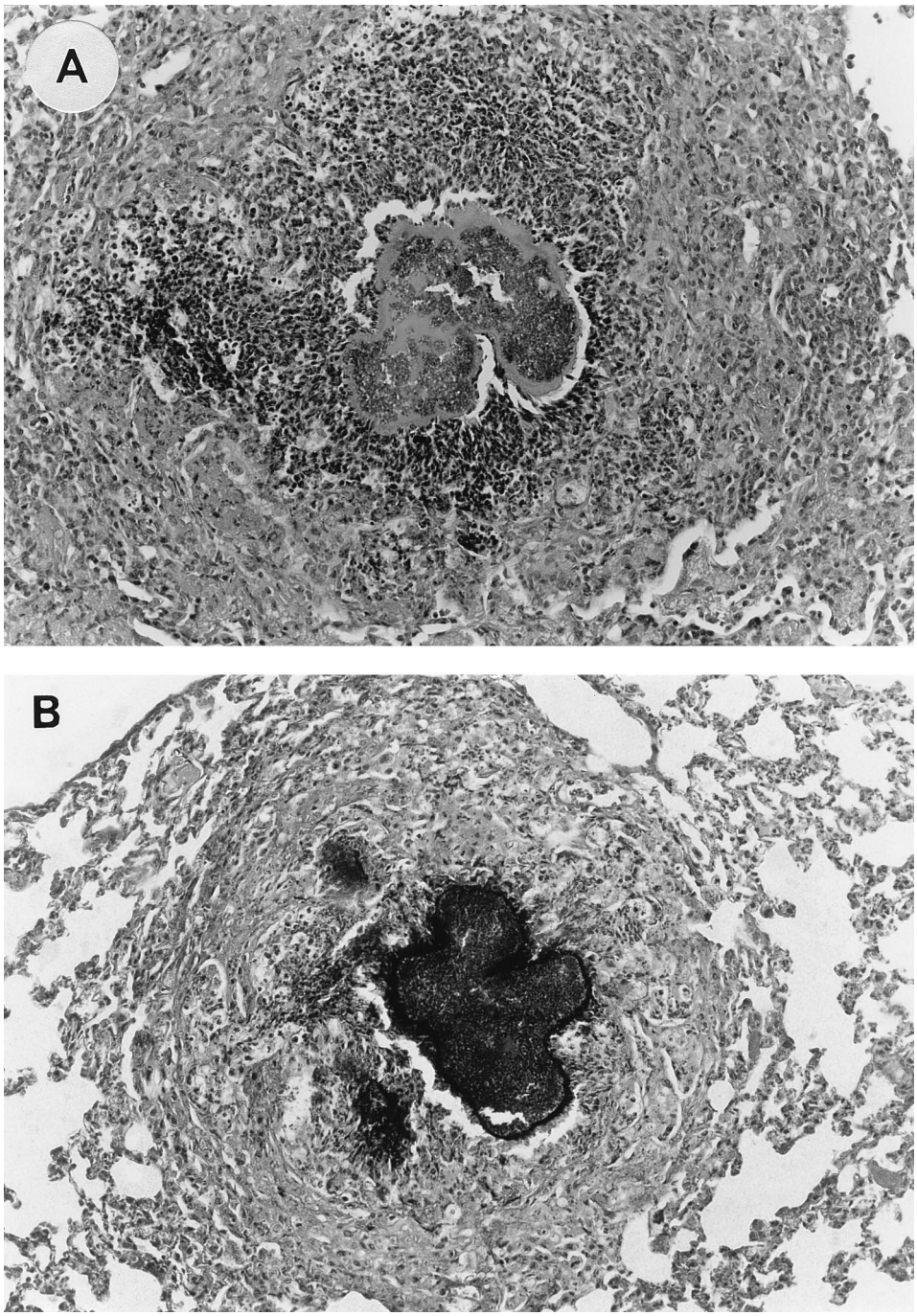


FIG. 2. (A) Microscopic examination of the lung 10 days after inoculation of bacteria. Note the lung abscess consisting of the central zone of the bacterial colony. Note also that the neutrophils and macrophages are separated from the bacterial colony by the layer surrounding the bacteria. Magnification, ×150. (B) Phosphotungstic acid-hematoxylin staining of the lung abscess demonstrating the presence of fibrin in the abscess. Note the linearly stained component between the colony of *S. aureus* and polymorphonuclear cells. The membrane probably consists of fibrin. Magnification, ×150.

the lung and other organs when the bacteria was suspended in saline. To our knowledge, this is the first animal model with reproducible and selective hematogenous lung abscess.

The pathological features of staphylococcal pneumonia produced experimentally in the mouse in the present study were similar to those in humans. Histological examination of the lung showed the presence of infective emboli in the peripheral pulmonary circulation, followed by bacterial proliferation and accumulation of inflammatory cells in blood vessels, and the spread of inflammation at a later stage to the lung, with abscess formation.

The virulence factors of *S. aureus* for proliferation in the tissue of mouse have been examined. The present study showed that the number of viable bacteria recovered from the lungs correlated with the titer of coagulase ($P < 0.01$). Furthermore, histological examination of the lung tissue showed

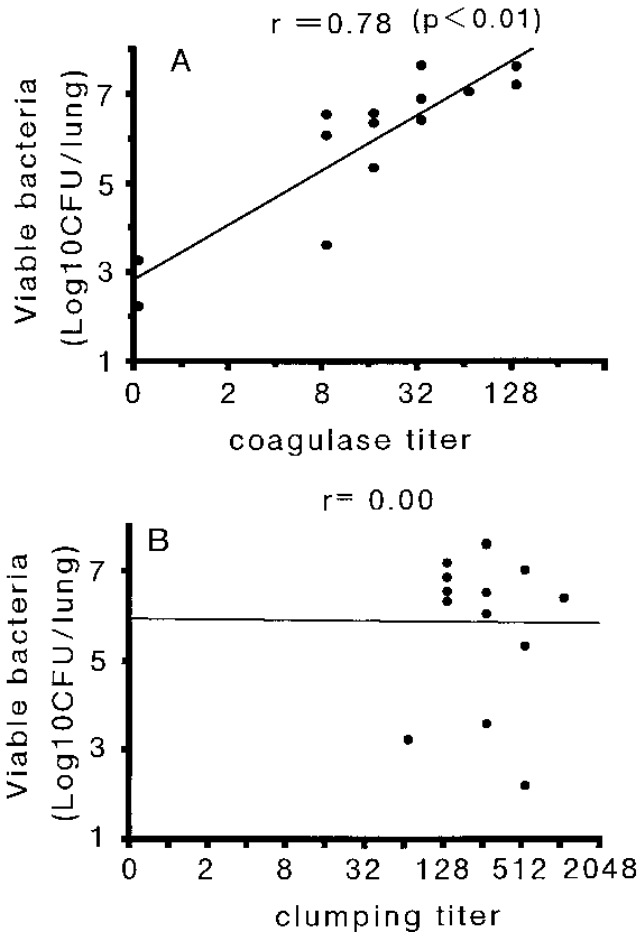


FIG. 3. Correlation between coagulase titer (A) or titer of clumping factor (B) of 14 strains of *S. aureus* and the number of viable bacteria isolated from the lung on day 7 after inoculation.

that the bacterial colonies inside the abscess were individually surrounded by a thin layer stained with phosphotungstic acid-hematoxylin, probably representing a fibrin membrane. These results suggest that coagulase may facilitate the development of blood-borne staphylococcal pneumonia. Staphylocoagulase is an extracellular protein produced by *S. aureus* and has long been used to distinguish this species from the less-virulent staphylococci (coagulase-negative staphylococci), although the exact role of the enzyme in staphylococcal infections is still unclear. The mode of action of coagulase includes plasma clotting and fibrinogen-binding activity (2, 20). Staphylocoagulase binds with prothrombin to form a complex compound called staphylothrombin that can stimulate plasma clotting by converting fibrinogen to fibrin (14, 17). It is probable that the formation of fibrin through the action of coagulase may enhance the resistance of *S. aureus* against phagocytosis; consequently, the multiplying bacteria may overcome the local phagocytic mechanisms. In this regard, several studies have reported that coagulase-negative mutants are significantly less virulent than the parental coagulase-positive strain when tested in mice (12, 13, 15, 19, 27); on the other hand, some other researchers proclaimed that differences in the virulence were not observed between the coagulase-deficient mutant and its parental strain (1, 4, 29). In all cases, the mutants were isolated following chemical mutagenesis; thus, the loss of virulence may be due to mutations affecting other factors (26). Moreillon et al. (21) reported, with a rat endocarditis model with coagulase-negative mutant that had undergone site-specific mutagenesis, that coagulase did not appear to function as a virulence factor. In contrast, the present experiments using strains of coagulase-positive and -negative site-specific mutants showed a significantly smaller number of coagulase-negative mutant strains at the site of infection.

TABLE 3. Comparison of the number of viable bacteria recovered from lung 7 days after inoculation with coagulase-positive *S. aureus* (DU5789) and coagulase-negative *S. aureus* (DU5843)

| Inoculum ($log_{10}$ CFU) | Bacterial count ($log_{10}$ CFU)[a] | |
|---|---|---|
| | DU5789[b] | DU5843[c] |
| 6.10 | 6.90 ± 0.26 | 4.80 ± 0.12[e] |
| 5.35 | 6.76 ± 0.35 | 4.96 ± 0.63[f] |
| 4.70 | 5.38 ± 0.41 | 2.73 ± 0.82[f] |
| 3.70 | 4.45 ± 0.73 | 1.87 ± 0.71[f] |
| 2.70 | 1.48 ± 0.48 | ND[d] |
| 1.70 | ND | ND |

[a] Values are means ± standard errors for five mice in each set of experiments.
[b] Coagulase titer, 128; clumping titer, 512.
[c] Coagulase titer, 0; clumping titer, 512.
[d] ND, not detected.
[e] $P < 0.01$ compared with mice injected with DU5789.
[f] $P < 0.05$ compared with mice injected with DU5789.

The controversy regarding the role of coagulase as a virulence factor may also be due to a difference in virulence related to attachment or proliferation of the bacteria. In the early experiments (21), animals were sacrificed 12 h after inoculation and examined for the role of coagulase in influencing the early establishment of valvular infection, promoting adherence or attachment to tissue. On the other hand, in the present experiments, animals were sacrificed 7 days after inoculation and then we investigated the role of coagulase at a later stage of infection, in promoting bacterial proliferation in the tissue.

Moreillon et al. (21) also reported that the clumping factor clearly affected bacterial adherence to the tissues. However, the role of clumping factor as a virulence factor was not established in our experiment. In our animal model, circulating bacteria enmeshed in agar beads after inoculation were physically trapped in the pulmonary microvasculature, similar to the infected thrombotic material causing septic pulmonary infarction in humans. Therefore, the action of the clumping factor on bacterial adherence to tissue is probably not important in blood-borne staphylococcal pneumonia. However, further investigation using clumping factor-deficient mutants is necessary in order to determine its virulence in a murine model of blood-borne pneumonia.

In conclusion, we described a novel and reproducible model of blood-borne staphylococcal pneumonia in a small laboratory animal. The model could be used for studies investigating the pathogenesis and therapy of *S. aureus*, involving methicillin-resistant *S. aureus*, and hematogenous pneumonia.

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