Gastrointestinal Colonization by Methicillin-Resistant *Staphylococcus aureus* in Immunosuppressed Mice

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ICR mice were inoculated intranasally with methicillin-resistant *Staphylococcus aureus* (MRSA) N133, and the inoculated MRSA was quantitatively recovered from the ceca and feces. The viable counts of the MRSA recovered from ceca correlated well with those from feces. Some mice eliminated MRSA from the cecum by 14 days after inoculation. Intraperitoneal administration of cyclophosphamide at a dose of 200 mg/kg 3 days before inoculation inhibited the elimination of the MRSA from both ceca and feces. All mice treated with cyclophosphamide excreted more than 10^4 CFU of the MRSA per g of feces for at least 70 days, indicating persistent colonization of the MRSA in the gastrointestinal tract. Some β -lactam antibiotics decreased the colonization level, but others did not. The colonization level was suggested to depend on the antibacterial activity of the antibiotic against the MRSA and the degree of disturbance of intestinal flora by the antibiotic.

Methicillin-resistant *Staphylococcus aureus* (MRSA) remains a life-threatening pathogen causing nosocomial infection in immunocompromised patients. Autoinfection from nasal carriage or cross-infection by staff members' hands seems to be the most common mode of acquisition of MRSA infections (4, 13). Gastrointestinal colonization with MRSA is regarded as a cause of MRSA infections because it occasionally proceeds to enterocolitis and toxic shock syndrome (10). The frequency of the incidence of MRSA enterocolitis has risen substantially as a result of the increase of patients with immunologic disorders and the wide use of broad-spectrum antibiotics (10, 15). Although oral administration of vancomycin was reported to be effective against MRSA enterocolitis (12), this disease is still associated with high mortality. Thus, it is important to prevent enterocolitis by eradicating colonized MRSA from gastrointestinal mucosa.

To define the factors involved in colonization, we established a gastrointestinal colonization model by intranasally inoculating immunosuppressed mice with MRSA and examined several antibiotics for their influence on colonization by MRSA.

MATERIALS AND METHODS

Bacterial strains. *S. aureus* N133 was obtained from the sputum of a patient. It is resistant to β -lactam antibiotics, kanamycin, and gentamicin.

S. aureus N133 produces type VII coagulase and does not produce enterotoxin and toxic shock syndrome toxin 1. The strain was stored at -80°C in Trypticase soy broth (Becton Dickinson and Company, Cockeysville, Md.) supplemented with 15% glycerol.

Animal model. Male specific-pathogen-free ICR mice (Crj:CD-1; Charles River Japan, Inc., Yokohama, Japan), aged 5 weeks, were used throughout the study. The animals were housed in cages with wire mesh bottoms to prevent secondary infection by coprophagia (6). The animals were given nonsterilized food pellets (CE-2; Japan Clea, Tokyo) and tap water ad libitum.

Immunosuppression was induced by intraperitoneally administering CY (Shionogi and Co., Ltd., Osaka, Japan), which was dissolved in distilled water at a dose of 200 mg/kg 3 days before inoculation.

MRSA inocula were prepared from organisms grown in 5 ml of brain heart infusion broth (Difco Laboratories, Detroit, Mich.) for 24 h at 37°C . Mice were anesthetized by intraperitoneal injection of 50 mg of sodium pentobarbital (Nembutal; Abbott Laboratories, North Chicago, Ill.) per kg of body weight and intranasally inoculated with 10^6 to 10^7 CFU of *S. aureus* N133 in 0.02 ml of brain heart infusion.

Antibiotic treatment. Ceftazidime (Nippon Glaxo Co., Ltd., Tokyo, Japan), imipenem-cilastatin (Banyu Pharmaceutical Co., Ltd., Tokyo, Japan), latamoxef (Shionogi and Co.), ceftizoxime (Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan), ampicillin (Meiji-Seika Kaisha, Tokyo, Japan), and cefazopran were used. All of the antibiotics except cefazopran were obtained from commercial sources. Cefazopran was synthesized at Takeda Chemical Industries, Ltd., Osaka, Japan. Antibiotics were dissolved in sterilized saline, and the solution was dispensed into 1.5-ml tubes and stored at -20°C until use. They were administered subcutaneously at a dose of 20 mg/kg twice a day (8:00 a.m. and 4:00 p.m.) for 3 days starting on the day after intranasal inoculation of MRSA.

Measurement of bacterial colonization. Gastrointestinal colonization by MRSA was verified by bacterial culture of ceca and feces. For the determination of bacterial count in the cecum, mice were killed by ether inhalation, and the cecum with contents was aseptically removed and homogenized with 3 ml of sterile water.

For the determination of bacterial count in feces, fresh feces were collected, weighed, and suspended in phosphate-buffered saline (PBS; Dulbecco formula [modified] without magnesium and calcium; Flow Laboratories, Inc., McLean, Va.) so that the final mixture contained 0.1 g of feces per ml. The suspension was vortexed for 2 min and diluted with PBS. In the case that fecal measurements and antibiotic administration were performed on the same day, feces were collected immediately before antibiotic administration.

The 0.1-ml volumes of cecal homogenates or fecal preparations were plated on mannitol salt agar (Eiken Chemical, Tokyo, Japan) containing 20 mg of gentamicin (Sigma Chemical, St. Louis, Mo.) and 20 mg of kanamycin (Meiji-Seika Kaishya) per liter. The fecal samples were plated within 3 h of evacuation. The detection limits of *S. aureus* N133 in cecum and feces were 16 CFU per cecum and 50 CFU per g of feces, respectively.

Measurement of fecal flora. Fecal bacteria were determined as described by Mitsuoka (14) and Summanen et al. (20). Fresh feces were collected, weighed, and suspended in the buffer described by Mitsuoka (14) so that the final mixture contained 0.1 g of feces per ml. Serial dilution and plating onto the plates for total anaerobes and *Clostridium difficile* were made in an anaerobic chamber.

Trypticase soy agar (Becton Dickinson) supplemented with 5% (vol/vol) sterile defibrinated horse blood was used to enumerate total aerobes. Staphylococci, enterococci, and enterobacteria were enumerated on mannitol salt agar (Eiken Chemical), EF agar (Nissui, Tokyo, Japan), and deoxycholate hydrogen sulfate lactose agar (Nissui), respectively. Brucella blood agar [brucella agar [Becton Dickinson] supplemented with 5% [vol/vol] sterile defibrinated sheep blood, 1 mg of vitamin K₁ [Wako Pure Chemical Industries, Ltd., Osaka, Japan] per liter, and 5 mg of hemin [Wako] per liter] was used to enumerate total anaerobes (20). Brucella blood agar supplemented with 50 mg of rifampin per liter was used for the selection of *Fusobacterium mortiferum*, *Fusobacterium varium*, and certain *Eubacterium* and *Clostridium* species (20). *Lactobacillus* spp. were enumerated on LBS agar (Becton Dickinson) supplemented with 8 g of Lablemco powder (Oxoid Ltd., Basingstoke, United Kingdom) per liter, 15 g of sodium acetate trihydrate (Wako) per liter, and 0.37% (vol/vol) acetic acid (Wako) (14). *Bacteroides* spp. and *C. difficile* were enumerated on bacteroides agar (Nissui) and cycloserine cefoxitin mannitol agar (Nissui), respectively.

All plates were incubated at 37°C . Mannitol salt agar plates and deoxycholate hydrogen sulfate lactose agar plates were incubated aerobically for 24 h. Trypticase soy agar plates and EF agar plates were incubated aerobically for 48 h. Brucella blood agar plates and cycloserine cefoxitin mannitol agar plates were incubated in an anaerobic chamber (90% N₂, 5% CO₂, 5% H₂) for 72 and 48 h,

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FIG. 1. Recovery of intranasally inoculated *S. aureus* N133 from the ceca of mice. ICR mice were inoculated intranasally with 9.6×10^6 CFU of *S. aureus* N133. The number of *S. aureus* N133 cells in the cecum was determined 1, 2, 3, 7, and 14 days after inoculation as described in Materials and Methods. Symbols: circles, bacterial counts in each mouse; solid line, geometric mean of bacterial counts in five mice; dotted line, limit of detection.

respectively. Bacteroides agar plates and brucella blood agar plates containing rifampin were incubated anaerobically in jars (Becton Dickinson) with Anaero Pack · Anaero (Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan) for 48 h. The LBS agar plates described above were incubated in a CO_2 incubator (5% CO_2) for 48 h. The detection limit was 10^2 CFU/g of feces.

Statistical analysis. Viable cell counts of *S. aureus* N133 and other bacteria in the feces were analyzed by the Dunnett type test. If no bacteria were recovered from the feces, the lower limit of detection was used for the calculations.

RESULTS

Detection of MRSA N133 in the cecum. For ease of anatomic identification, the cecum was chosen as a site of sampling. Anesthetized mice were inoculated intranasally with 9.6×10^6 CFU of *S. aureus* N133, and the bacterial counts in the cecum were examined as described in Materials and Methods. Only *S. aureus* N133 was recovered on mannitol salt agar containing gentamicin and kanamycin, and the recovery was quantitative. The number of *S. aureus* N133 cells recovered from the cecum was 10^3 to 10^4 CFU on the day after inoculation, decreased to 10^2 to 10^3 CFU 3 days after inoculation, and then slightly increased again (Fig. 1). *S. aureus* N133 was recovered from nasal wash fluid and lungs on the first 3 days but localized in the intestinal tract by 7 days after inoculation (data not shown). Fourteen days after inoculation, one of five mice eliminated *S. aureus* N133 (Fig. 1). We obtained a similar result in another experiment.

Recovery of MRSA from the cecum and feces. Viable cell counts of MRSA recovered from the cecum and feces were compared. Mice were inoculated intranasally with 3.2×10^6 CFU of *S. aureus* N133. Seven days after inoculation, the bacterial counts in the feces and cecum were examined. The mouse whose cecum was free of *S. aureus* N133 did not excrete the organism in its feces. The viable cell counts of *S. aureus* N133 recovered from the cecum correlated well ($r = 0.886$) with those from the feces (Fig. 2a). This result indicates that the colonization level in the gastrointestinal tract can be estimated by measuring the cell counts in feces. For this reason, the number of MRSA cells in the feces was counted in all the remaining experiments.

Influence of immunosuppressant. We have previously shown that immunosuppression promoted the growth of MRSA in the mouse cecum (9). Therefore, we examined the influence of an immunosuppressant on gastrointestinal colonization. Mice were administered CY intraperitoneally at a dose

FIG. 2. Relationship between viable cell counts of *S. aureus* N133 in the cecum and feces (a) and effect of CY on the viable cell counts in feces (b). Normal (A) (open circles) and CY-treated (B) (closed circles) mice were inoculated intranasally with 3.2×10^6 CFU of *S. aureus* N133. CY was administered intraperitoneally at a dose of 200 mg/kg 3 days before inoculation. Seven days after inoculation, viable cell counts of MRSA N133 in cecum homogenate and fecal suspension were determined. Symbols: circles, bacterial counts in each mouse ($n = 20$); dotted lines, limits of detection; solid line in panel a, regression line ($r = 0.886$); horizontal bars in panel b, mean values.

of 200 mg/kg to suppress immunity. From 1 to 7 days after CY injection, atrophy of Peyer's patches was observed. An example of the atrophy of Peyer's patches 4 days after injection is shown in Fig. 3.

In CY-treated mice, the number of *S. aureus* N133 cells in feces did not decrease during the first 3 days after inoculation, in contrast to normal mice (Fig. 4). Seven days after inoculation, the cell level of *S. aureus* N133 in feces from CY-treated mice was higher than that from normal mice (Fig. 2b). Cortisone acetate administered subcutaneously on the day before inoculation at a dose of 100 mg/kg had a similar effect (data not shown). At least until 70 days after inoculation, all mice treated with CY excreted more than 10^4 CFU of *S. aureus* N133 per g of feces without diarrhea (data not shown). These findings indicate that CY pretreatment enhances gastrointestinal colonization by *S. aureus* N133.

Influence of β -lactam antibiotic treatment. We investigated the influence of β -lactam antibiotics on intestinal colonization by MRSA since antibiotic administration is suspected to increase MRSA colonization (10, 15). Viable cell counts in the

FIG. 3. Intestines of normal (upper) and CY-treated (lower) mice. CY was administered intraperitoneally at a dose of 200 mg/kg 4 days before abdominal examination. Arrows indicate Peyer's patches.

FIG. 4. Fecal excretion of *S. aureus* N133 in normal (A) and CY-treated (B) mice. Normal and CY-treated mice were inoculated intranasally with 4.5×10^6 CFU of *S. aureus* N133. CY was administered intraperitoneally at a dose of 200 mg/kg 3 days before inoculation. Solid lines connect the results for individual mice.

feces of antibiotic-treated mice were determined every day until 7 days after inoculation (Fig. 5). The level of *S. aureus* N133 in the feces of mice which received saline remained unchanged. The number of *S. aureus* N133 cells decreased in most of the mice which received antibiotics. In mice treated with cefazopran, viable cell counts of *S. aureus* N133 in the feces continued to decrease even after the cessation of administration, and by 7 days after inoculation, MRSA was cleared from all mice except one that had an extraordinary large number of MRSA at the beginning. In mice treated with ceftazidime, the decreases in the cell number were slight. In two imipenem-treated mice, the cell count decreased to below 10^2 CFU/g but increased again by 7 days after inoculation.

The effect of treatment with cefazopran and imipenem on the number of facultative anaerobes and anaerobes in the feces was investigated (Table 1). Imipenem led to an increase in the number of *Enterococcus* spp. and *S. aureus* N133 to 10^7 to 10^{10} CFU/g and 10^3 to 10^5 CFU/g of feces, respectively. *C. difficile*, a cause of antibiotic-associated pseudomembranous colitis, was detected in two of five mice which received imipenem. Both antibiotics reduced the number of *Staphylococcus* and *Lactobacillus* spp. The number of enterobacteria decreased in cefazopran-treated mice, whereas it tended to increase in imipenem-treated mice. The number of anaerobes did not change significantly. The level of each bacterium except *S. aureus* N133 returned to the pretreatment level 5 days after final antibiotic administration (data not shown).

Seven days after intranasal inoculation of *S. aureus* N133, the feces of the mice given cefazopran contained substantially lower numbers of *S. aureus* N133 than those of control mice ($P < 0.01$) (Fig. 6). The feces of four of five mice were free of MRSA. No decrease in viable cell counts was observed in the groups treated with other antibiotics.

DISCUSSION

Nasal carriage was reported to be a cause of nosocomial infection of MRSA (4, 13). To represent the clinical situation, we chose the intranasal route of inoculation to induce gastrointestinal colonization by MRSA. When *S. aureus* N133 was inoculated orally, the organism was eliminated from the ceca of all unanesthetized mice and three of five anesthetized mice by 7 days after inoculation (data not shown).

We first tried to establish a chronic gastrointestinal coloni-

FIG. 5. Influence of three antibiotics on the fecal level of intranasally inoculated *S. aureus* N133. CY-treated mice were inoculated intranasally with 1.7×10^6 CFU of *S. aureus* N133 and received saline (A), cefazopran (B), ceftazidime (C), or imipenem (D) subcutaneously at the times indicated by arrowheads. The dose of antibiotics was 20 mg/kg. MICs of the antibiotics for *S. aureus* N133 are described in the legend to Fig. 6. Solid lines connect the results for individual mice.

zation model by using *S. aureus* N133 and normal mice. However, it was difficult to induce colonization in all of the mice. Some mice cleared *S. aureus* N133, while others maintained it at various levels (Fig. 1 and 2b). Ida et al. (11) reported that preexisting methicillin-susceptible *S. aureus* competed with inoculated MRSA for intestinal colonization. All mice used in this study, however, excreted methicillin-susceptible *S. aureus* in the feces before and even after MRSA inoculation (data not shown), indicating that competition with methicillin-susceptible *S. aureus* was not a cause of incomplete colonization of MRSA in our experiment.

TABLE 1. Effect of ceftazidime and imipenem on fecal microflora of immunosuppressed mice

Fecal microflora	Log CFU/g of feces ^a		
	Before inoculation (n = 10)	After administration of:	
		Ceftazidime (n = 5)	Imipenem (n = 5)
Total aerobes	9.00 ± 0.42	8.07 ± 0.62	9.01 ± 1.34
<i>Staphylococcus</i> spp.	5.37 ± 0.49	3.04 ± 0.20*	4.17 ± 0.86*
<i>S. aureus</i> N133	<2.00 ^b	2.17 ± 0.38	4.16 ± 0.84*
<i>Enterococcus</i> spp.	6.37 ± 0.61	6.22 ± 1.35	8.51 ± 1.23*
Enterobacteria	6.67 ± 0.74	4.18 ± 1.08*	7.28 ± 1.71
<i>Lactobacillus</i> spp.	9.17 ± 0.45	5.50 ± 1.71*	5.85 ± 1.73*
Total anaerobes	9.94 ± 0.23	9.67 ± 0.61	10.38 ± 0.75
<i>Bacteroides</i> spp.	9.33 ± 0.34	8.32 ± 1.20	9.07 ± 0.97
Anaerobic rods ^c	7.64 ± 0.74	7.90 ± 0.53	8.06 ± 0.65
<i>C. difficile</i>	<2.00 ^b	2.00 ^b	3.25 ± 1.85

^a Geometric mean ± standard deviation of the bacterial counts per gram of feces. Fecal bacteria were counted just before inoculation of *S. aureus* N133 (3 days after CY injection) and on the day after the final administration of ceftazidime and imipenem. *, significantly different from the value before inoculation ($P < 0.01$).

^b Not detected in feces from any mouse.

^c *Fusobacterium mortiferum*, *Fusobacterium varium*, and certain *Eubacterium* and *Clostridium* species.

The influence of an immunosuppressant on gastrointestinal colonization was examined next. Intraperitoneal injection of 200 mg of CY per kg induced atrophy of Peyer's patches (Fig. 3), indicating that the immune response in gut-associated lymphoid tissue was suppressed. The number of *S. aureus* N133 cells in the feces of the mice treated with CY was retained at a high level for the first 3 days (Fig. 4), resulting in chronic colonization in all mice. CY produced high *Candida* counts in the ceca of ICR Swiss mice (6) and enhanced the susceptibility of SCID mice to mucosal candidiasis (3). CY induced a transient decrease in the T- and B-cell populations in the Peyer's

patches and a profound decrease in intestinal immunoglobulin A secretion as a result of a reduction of lamina propria plasma cells (5). It has also been shown that CY inhibited the growth of the T-cell population in Peyer's patches of MRL1/1 female mice (26). Thus, it is considered that CY induced the colonization of *S. aureus* N133 by suppressing immune responses, although further studies are necessary to define the immunologic factors involved in mucosal colonization.

Gastrointestinal colonization by *S. aureus* N133 was not lethal for ICR mice. Another MRSA strain, which produced enterotoxin C and toxic shock syndrome toxin 1, caused neither diarrhea nor symptoms of systemic infection after gastrointestinal colonization (data not shown). However, several studies (7, 8, 25) have linked antibiotic administration or immunosuppression with intestinal overgrowth and translocation of bacteria into extraintestinal sites. Repeated antibiotic administration at a higher dose or additional CY injection may be necessary for the overgrowth and translocation of MRSA in our model.

It should be noted that colonization resistance (21, 23) is provided by factors other than immunity. It is mediated mainly by obligate anaerobic microorganisms and also by salivation, swallowing, secretion of immunoglobulin A, production of gastric acid, desquamation of cells of the mucous membranes, and normal gastrointestinal motility (21). Administration of broad-spectrum antibiotics was associated with an increase in the concentration of potentially pathogenic microorganisms in the gastrointestinal lumen by destruction of colonization resistance (1, 16–19, 22).

Administration of antibiotics, however, resulted in the reduction of *S. aureus* N133 during antibiotic administration (Fig. 5). The most-prominent reduction was observed in mice treated with ceftazidime, and the least-prominent reduction was observed in mice treated with ceftazidime, which had a lesser antibacterial activity than ceftazidime. The decrease in the number of *S. aureus* N133 cells during antibiotic administration must be due to the direct influence of the antibiotics on the bacteria.

In four ceftazidime-treated mice, the number of *S. aureus* N133 cells in the feces continued to decrease even after the final administration. On the other hand, the level of *S. aureus* N133 in two mice, which had dropped below 10^2 CFU/g after the administration of imipenem, increased again by 7 days after inoculation (Fig. 5). The intestinal flora is considered to play some role in reducing the number of *S. aureus* N133 cells after the cessation of antibiotic administration. Samonis et al. (16–19) indicated that antibiotics which had potent activity against anaerobic bacteria or high gastrointestinal concentration promoted gastrointestinal colonization by yeasts to a greater extent than did antibiotics with poor activity against anaerobic bacteria or low gastrointestinal concentration. Ceftazidime, which is excreted mainly in urine, had milder activity (2, 24) than ampicillin, latamoxef, and imipenem against obligate anaerobic microorganisms, which consist of protective flora, and provided the least effect on mouse intestinal flora (24). Imipenem substantially increased the population of some aerobes, especially *Enterococcus* spp., in the fecal flora, although ceftazidime did not (Table 1). The overgrowth of *Enterococcus* spp. presumably indicated a collapse of bacterial balance in the intestinal flora and a decrease in colonization resistance. Regrowth of *S. aureus* N133 in imipenem-treated mice (Fig. 5) may be due to these changes in bacterial balance. Although we were not able to specify the bacteria which were responsible for maintaining colonization resistance, keeping a bacteriological balance in the intestinal flora seemed to be necessary to prevent colonization by MRSA.

FIG. 6. Influence of antibiotics on gastrointestinal colonization by *S. aureus* N133 in mice. Mice pretreated with CY were inoculated intranasally with 1.1×10^6 CFU of *S. aureus* N133. Antibiotics were administered subcutaneously at a dose of 20 mg/kg twice a day for 3 days starting on the day after inoculation. Seven days after inoculation, the viable cell counts of MRSA in the feces were determined. The antibiotics used were ceftazidime (CZOP; 25 µg/ml), ceftazidime (CAZ; >100 µg/ml), imipenem-cilastatin (IPM; 25 µg/ml), latamoxef (LMOX; >100 µg/ml), ceftazoxime (CZX; >100 µg/ml), and ampicillin (ABPC; 25 µg/ml) (the concentrations given in parentheses are the MICs for *S. aureus* N133). Bacterial counts less than the detection limit (50 CFU/g of feces) were regarded as 50 CFU to calculate the means. Error bars indicate standard deviations in five mice. **, significantly different from the value for control mice which received saline ($P < 0.01$).

We indicated that both intestinal immunity and bacterial flora were concerned with protection against gastrointestinal colonization by MRSA. The MRSA colonization model established here will be useful for analyzing the mechanism of MRSA colonization in the intestinal tract and the effect of antibiotics on colonization.

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