Positive Reaction in Mouse Ligated Intestinal Loop Assay with Nonenterotoxigenic and Nonhemolytic Strains of Staphylococcus aureus

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Nonenterotoxigenic and nonhemolytic strains of Staphylococcus aureus exhibited a positive reaction in the mouse intestinal loop assay except for a strain negative for the egg yolk reaction. Edema and swelling in all positive loops, increased bacterial cell numbers within intestinal loops, and extremely close adhesion to or inclusion of numerous bacterial cells in HeLa cells after inoculation of the strains were observed. These results suggest a possible invasive ability of the strains.

Invasive properties of Staphylococcus aureus strains have been recognized only in unusually severe infections (3). These organisms are also assumed to be capable of inducing cellular immunity (12), suggesting a possible invasive ability of the organisms. The ligated intestinal loop assay in rabbits was first reported by De and Chatterjee (4) as a new model for the investigation of infection. This method was performed in mice by Punyashthiti and Finkelstein (9). With a further modification of this technique in mice, we observed positive reactions in the mouse ligated intestinal loop assay with nonenterotoxigenic and nonhemolytic strains of S. aureus. In addition, findings suspicious of invasion were shown in a HeLa cell assay. This paper describes these experimental results.

Strains SMU170, SMU171, SMU460, SMU141, SMU364, SMU001, and SMU101 of S. aureus isolated from clinical specimens in the Bacteriology Section, St. Marianna University Medical School Hospital, were used. All strains were positive for coagulase, D$	ext{N}$ase, catalase, and phosphatase and produced no hemolysin on 5% sheep blood agar (BBL Microbiology Systems, Cockeysville, Md.). Except for strain SMU170, they were positive for the egg yolk reaction when examined by the method of Tirunarayanan and Lundbeck (11). Also, they produced no enterotoxin except for strain SMU101, which produced enterotoxin A as determined with a staphylococcal enterotoxin detection kit (Denka Seiken Co., Ltd., Tokyo, Japan) which is similar to that of Oxdid (SET-RPLA; Oxdid Ltd., London, England). Staphylococcal strains grown in brain heart infusion (Difco Laboratories, Detroit, Mich.) broth at 37°C overnight were washed once with saline by centrifugation, and cell suspensions exhibiting 1.0 unit of optical density at 380 nm were nephelometrically prepared with brain heart infusion broth. The viable cell numbers were 2.06 × 10^9 to 3.24 × 10^9 CFU/ml, enumerated by a regular pour plate count method.

The mouse intestinal loop assay was performed by the method of Punyashthiti and Finkelstein (9) modified as follows. A group of four to five mice (JCL strain; ICR; Nihon Clea Farm, Tokyo, Japan) weighing approximately 30 g and inbred for a long period of time in our laboratory were injected intraperitoneally with 3 mg of cyclophosphamide in 0.5 ml of saline. After 3 days, the intestinal loop assay was performed by the method of Punyashthiti and Finkelstein (9) with the above-described cell suspension. Two independent 2-cm intestinal loops were separated from each mouse, and 0.2 ml of bacterial cell suspension was injected into one of the loops and another sample or brain heart infusion broth alone was injected into the other. Eighteen hours later, macroscopic findings in the ligated intestinal loops, such as edema and swelling, were observed and the length of the loop was measured. Then the loop was opened, the volume of fluid was quantitated, and the weight of fluid in the loop per centimeter of gut length was calculated. When the weight of fluid in the loops was greater than 50 mg/cm, they were considered positive, according to Punyashthiti and Finkelstein (9). For the enumeration of viable cells within the ligated intestinal loops, the lumen of the loop was washed several times with 0.067 M phosphate-buffered saline (pH 7.2) to remove adherent organisms from the surface of the mucosa. Then the loop was homogenized with a homogenizer (Nihon Seiki Co., Ltd., Tokyo, Japan) in brain heart infusion broth, and viable cells were counted by the regular pour plate count method.

The effect of staphylococcal cells on HeLa cells was determined by the modified method of Gerber and Watkins (6) and LaBrec et al. (8) as follows. Monolayer HeLa cells were cultured in Eagle minimal essential medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 5% fetal bovine serum (GIBCO Laboratories, Grand Island, N.Y.). A 0.4-ml sample of the HeLa cell suspension containing 10^4 cells per ml was dispensed onto tissue culture slides (Miles Scientific, Div. Miles Laboratories, Inc., Elkhart, Ind.) consisting of eight chambers and cultured at 37°C for 48 h. Then, 5 μl of bacterial cell suspension was added to each chamber. After incubation at 37°C for 2 h, the cells were washed three times with the broth medium and further cultured for 6 h in the same medium. They were washed gently with phosphate-buffered saline, fixed with methanol, stained with 2% Giemsa solution, and examined by light microscopy.

In the mouse intestinal loop assay, four of five loops (80.0%), three of five loops (60.0%), three of five loops (60.0%), four of five loops (80.0%), three of four loops (75.0%), and all of four loops (100%) of strains SMU171,
enterotoxin A showed a positive reaction, corroborating the data of Koupal and Deibel (7). In the present experiments, macroscopically recognizable edema and swelling were observed in all positive loops. Increasing numbers of viable cells were seen in these loops, and numerous bacterial cells were found in the epithelial cell fraction. HeLa cells inoculated with these strains showed cytopathologic alterations, and a number of bacterial cells appeared to be within the cells, although some of them might simply have been closely adhering to the surface of altered HeLa cells. All these findings suggest the possibility of invasive properties for the strains used in these experiments, although such activity by S. aureus strains has been regarded as unusual (3).

Concerning the factors responsible for the positive intestinal loop assay, it was speculated that the egg yolk factor might play an important role. However, the egg yolk reaction is basically considered to be related to lipase or lipase plus phosphatase, and no relationship to pathogenicity has been recognized for these enzymes. The question of whether or not S. aureus possesses truly invasive ability is crucial in terms of investigating infective and immune aspects of these organisms. To elucidate these questions, further investigations are required.

LITERATURE CITED


FIG. 1. Recovery of six strains of S. aureus from the intestinal cavities of mice treated with cyclophosphamide.

SMU141, SMU460, SMU364, SMU001, and SMU101, respectively, showed positive reactions. In particular, the mean fluid weights per gut length of loops treated with strains SMU171 and SMU460 were 110.6 and 98.5 mg/cm, respectively, and a loop inoculated with strain SMU171 reached 300 mg/cm. Also, all positive loops exhibited edema and swelling. However, with strain SMU170, which was negative for the egg yolk reaction, none of the loops showed positive results. Upon enumeration of viable cells at 18 h after inoculation of the organisms into the loops, every strain, except for strain SMU170, showed a significantly higher number of viable cells in loop homogenates and fluid (Fig. 1), although no staphylococcal cells were detected in control loops inoculated with brain heart infusion broth only. In the HeLa cells inoculated with staphylococcal strains, a majority of cells exhibited slight to moderate grades of cytopathologic morphological alterations and numerous bacterial cells were closely adherent to or contained within the cells even after washing.

The mechanisms by which a positive reaction occurs in ligated intestinal loops differ depending on the pathogenic bacterial strains. With cholera bacilli, the positive reaction is caused by toxin (2, 5), whereas bacterial invasion into the mucosa of the small intestine causes a positive reaction with Shigella strains (1, 10). Recently, Koupal and Deibel (7) noted positive reactions in the rabbit intestinal loop assay with enterotoxigenic strains of S. aureus.

In the present experiments, when mice were treated with cyclophosphamide, the intestinal loop assay demonstrated a positive reaction with all nonenterotoxigenic and nonhemolytic strains of S. aureus except for a strain which was negative for the egg yolk reaction. A strain producing