J Orthop Sci (1998) 3:95–101

Journal of
Orthopaedic Science

Detection of human serum antibody to encapsulated strains of *Staphylococcus aureus* by enzyme-linked immunosorbent assay inhibition test*

Hiroki Ueda[1], Kazuhiko Matsushita[1], and Yoshitoshi Ichiman[2]

[1] Department of Orthopaedic Surgery, St. Marianna University School of Medicine, 2-16-1, Sugao, Miyamae-ku, Kawasaki 216, Japan
[2] Department of Hygiene and Public Health, Tsukuba College of Technology, 4-12-7, Kasuga, Tsukuba 305, Japan

Abstract: A specific and rapid enzyme-linked immunosorbent assay (ELISA) inhibition test was employed for detection of immunoglobulins to *Staphylococcus aureus* (*S. aureus*) capsular polysaccharide in human serum. Capsular polysaccharide antigens obtained from Smith diffuse (capsular type 2), Reynolds (capsular type 5), or Becker (capsular type 8) strains of *S. aureus* were added to microplates coated with these strains. Seventy-four patients with open fractures (31 serum samples from those with staphylococcal infections, 10 serum samples from those with non-staphylococcal infections, and 33 serum samples from the non-infected group) and 28 serum samples from healthy controls were then added. The plates were incubated at 37°C for 2 h and the ELISA was performed. The ELISA inhibition assay showed remarkable inhibition with the capsular type 2, 5, and 8 polysaccharides in the 33 serum samples from the non-infected group and in the 28 serum samples from the healthy controls, but low inhibition was observed with the 31 sera with staphylococcal infections. Positive immunoglobulin (Ig)G and IgM titers showed marked inhibition with this assay, but IgA titer were not seen in any samples. These results indicate that the quantitation of human serum antibody against *S. aureus* capsular polysaccharide by the ELISA inhibition assay is useful for the demonstration of protective activities against *S. aureus*.

Key words: bone and joint infections, *Staphylococcus aureus*, antibody, enzyme-linked immunosorbent assay (ELISA)

Introduction

Open wounds in orthopedic surgery frequently result in infections of the soft tissue, bone, and joints, even when appropriate initial treatment is given. The most common causative organism of such infections is *Staphylococcus aureus*.[19,20] These infections are usually treated with antibiotics[10] or by surgery,[1,12] but they are sometimes refractory.[17,20] In the field of orthopedic surgery, to our knowledge, there have been no reports on antibody carrier status for various types of bacteria in the host or on the role of subsequent therapy in such infections.

We studied the presence of protective antibodies against infections with encapsulated *S. aureus* in the serum of patients and healthy individuals as one means to clarify the in vivo defense mechanism in human serum.

Materials and methods

Bacterial strains

Smith (Fig. 1), Reynolds, and Becker *S. aureus* strains were used as representative strains of capsular types 2, 5, and 8, respectively.

Preparation of capsular polysaccharide

The capsular polysaccharide was extracted from the Smith, Reynolds, and Becker strains by a modification of the method of Morse.[13] Strains were grown on Brain Heart Infusion (BHI; Difco, Detroit, MI, USA) dialysate in agar medium at 37°C for 18 h, after which they were harvested, washed once with saline, and then resuspended in saline. These suspensions were subjected to sonic oscillation (UR200p; Tominaga, Tokyo,

Offprint requests to: H. Ueda
Received for publication on June 6, 1997; accepted on Sept. 12, 1997
*This study was outlined at the 20th Meeting of the SIROT, the 45th Meeting of the Eastern Japan Association of Orthopaedics and Traumatology, and the 19th Annual Meeting of Japanese Society for Study of Bone and Joint Infections

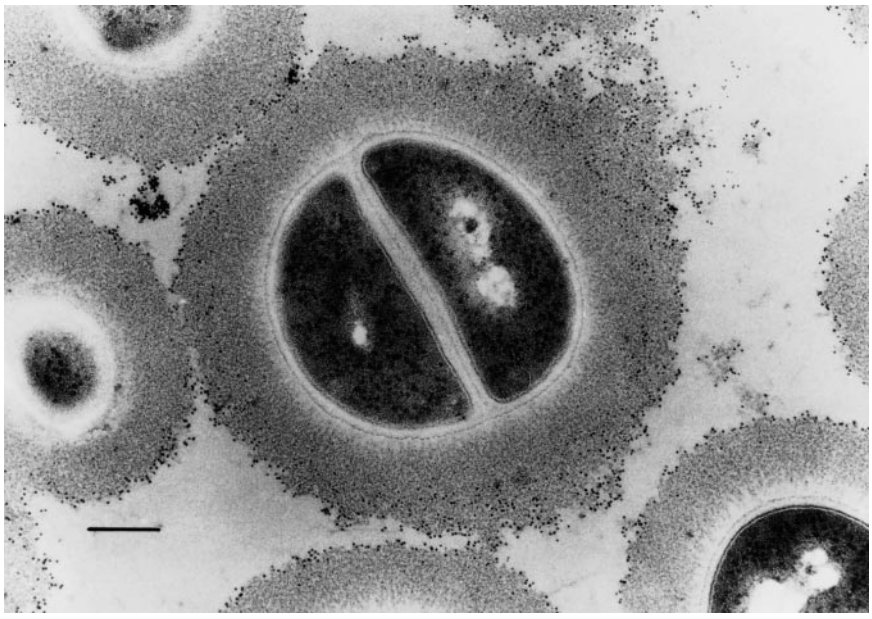

Fig. 1. Electron micrograph of Smith strain of *Staphylococcus aureus* treated with rabbit anti-Smith strain serum conjugated with ferritin. *Scale bar*, 0.1μm

Japan) at 10 kilocycles for 5 min, after which the organisms were removed by centrifugation at 8000 rpm for 20 min. Then, 30 μg of RNase and 10 μg of DNase were added to the fractions, which were treated with enzyme for 30 min. Deproteination was performed with chloroform, and the precipitate obtained by adding a fivefold volume of ethanol to the solution was submitted to delipidation with ether and acetone. The substance obtained on overnight drying in a vacuum cryodessicator (Shimadzu, Kyoto, Japan) was the capsular polysaccharide.

Human serum

The sera used were obtained from 74 patients with open fractures or wounds hospitalized in the Department of Orthopedic Surgery, St. Marianna University School of Medicine from April to December 1995, and from 28 healthy controls. The 74 inpatients underwent bacteriological examinations of the wound during hospitalization and were grouped according to the bacteria detected. Group 1 consisted of 31 patients in whom *S. aureus* was detected, including methicillin-resistant *S. aureus* (MRSA). Group II consisted of 10 patients in whom bacteria other than *S. aureus* were detected, and group III consisted of 33 patients with wounds negative for bacteria. Group IV (controls) consisted of 28 students at St. Marianna University School of Medicine with no history of severe systemic bacterial infections. Serum was collected from each group.

Enzyme-linked immunosorbent assay (ELISA) inhibition test

According to the method of Ichiman et al.,[7] 5 μg of each of the capsular polysaccharides extracted from each strain of bacteria and dissolved in 50 μl of phosphate-buffered saline (PBS, pH 7.5) was placed in a 96-well microplate and allowed to stand at 4°C for 24 h. Then, the plate was washed four times with PBS (pH 7.2) plus 0.1% Tween 80. After treatment of the plate with 1% bovine serum albumin, 1 μg of capsular polysaccharide dissolved in 25 μl of PBS was added, 25 μl of human serum diluted 100-fold was immediately added, and the mixture was reacted for 2 h at room temperature (a plate with no capsular added polysaccharide was used as control). The plate was again washed four times with PBS (pH 7.2) plus 0.1% Tween 80, and alkali phosphatase-labeled anti-human immunoglobulin (Ig)G, IgM, or IgA (Sigma, St. Louis, MO, USA) was added. The plate was left to stand for 2 h at room temperature, and was then washed four times with PBS (pH 7.2) plus 0.1% Tween 80; P-nitrophenylphosphate was then added and the mixture was reacted for 30 min at room temperature. The colored end-product was measured spectrophotometrically at 405 nm with an enzyme immunoassay (EIA) reader (Bio-Rad Laboratories, Hercules, CA, USA). The ELISA inhibition rate (%) was obtained with the equation:

[1 − ELISA value of human serum with no capsular polysaccharide added (OD)/ELISA value of human serum with capsular polysaccharide added (OD)] × 100

Thus, the higher the value of the ELISA inhibition rate given by the equation, the higher was the antibody content for the capsular polysaccharide extracted from each strain.

Statistical analysis

The numerical values obtained were expressed as means ± SD. Significant intergroup differences were tested by the Kruskal-Wallis test, and P values of 0.05 or less were taken as significant differences.

Results

Mean inhibition rates of antibody activity for the Smith strain of S. aureus *in human serum*

IgG. The mean inhibition rates for IgG were 14.9 ± 10.6% in group I, 19.2 ± 8.2% in group II, 35.7 ± 13.2% in group III, and 41.9 ± 12.6% in group IV. The values were higher in groups III and IV than in groups I and II. There were significant differences between groups I and III and between groups I and IV and between groups II and IV ($P < 0.01$) (Fig. 2).

IgM. The mean inhibition rates for IgM were 5.9 ± 7.2% in group I, 11.2 ± 10.8% in group II, 20.6 ± 10.6% in group III, and 21.4 ± 10.3% in group IV. The values were higher in groups II, III, and IV than in group I. There were significant differences between groups I and III and between groups I and IV ($P < 0.01$) (Fig. 3).

IgA. The mean inhibition rates for IgA were 1.2 ± 1.9% in group I, 2.0 ± 4.1% in group II, 2.2 ± 3.3% in group III, and 2.1 ± 2.7% in group IV. There were no significant differences between any of the groups (Fig. 4).

Mean inhibition rates of antibody activity for Reynolds strain of S. aureus *in human serum*

IgG. The mean inhibition rates for IgG were 41.7 ± 11.4% in group I, 52.6 ± 9.4% in group II, 50.2 ± 8.5% in group III, and 57.0 ± 11.5% in group IV. The values were higher in groups II, III, and IV than in group I. There was a significant difference between groups I and IV ($P < 0.01$) (Fig. 5).

IgM. The mean inhibition rates for IgM were 13.0 ± 8.1% in group I, 17.0 ± 6.5% in group II, 22.8 ± 11.1% in group III, and 24.1 ± 8.4% in group IV. The values were higher in groups III and IV than in groups I and II. There were significant differences between groups I and III and between groups I and IV ($P < 0.01$) (Fig. 6), as for the IgG values.

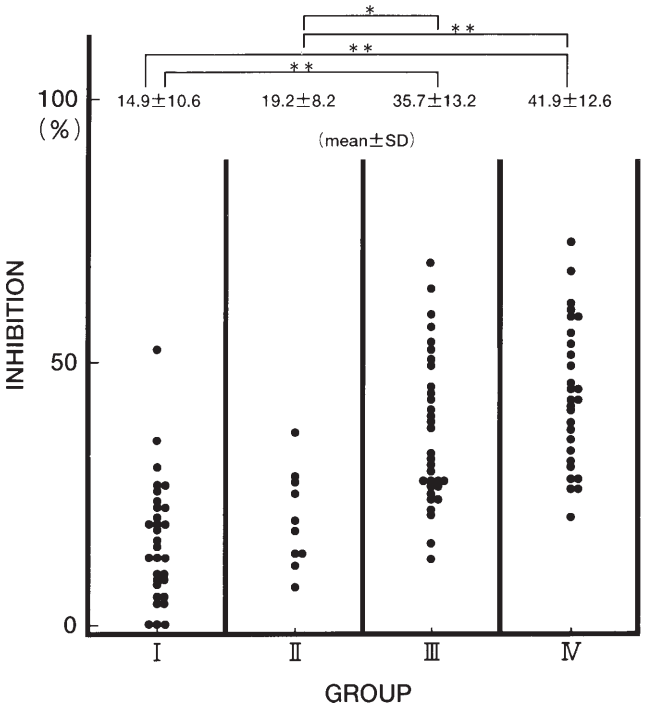

Fig. 2. Immunoglobulin (Ig)G antibody in human serum against capsular polysaccharide extracted from Smith strain of *Staphylococcus aureus* by enzyme-linked immunosorbent assay inhibition test. *$P < 0.05$; **$P < 0.01$. *Group I*, *S. aureus*-positive patients; *group II*, patients positive for bacteria other than *S. aureus*; *group III*, patients negative for bacteria; *group IV*, healthy controls

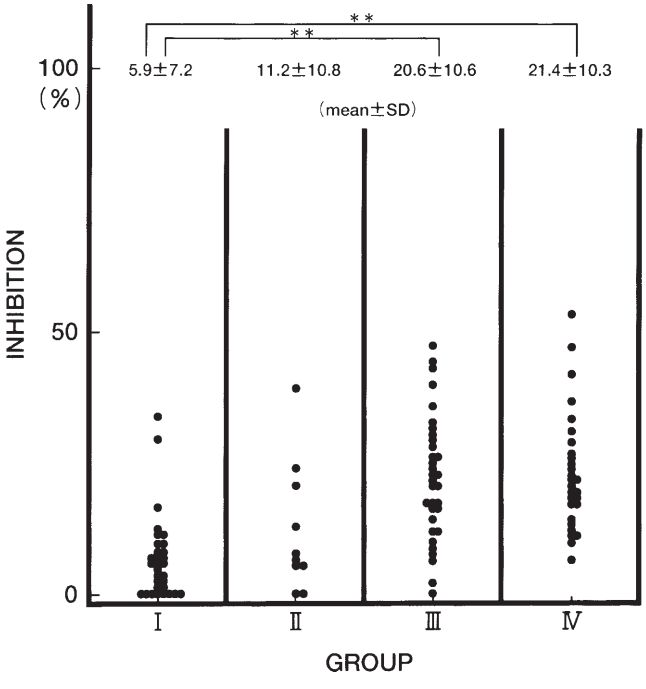

Fig. 3. IgM antibody in human serum against capsular polysaccharide extracted from Smith strain of *Staphylococcus aureus* by enzyme-linked immunosorbent assay inhibition test. **$P < 0.01$

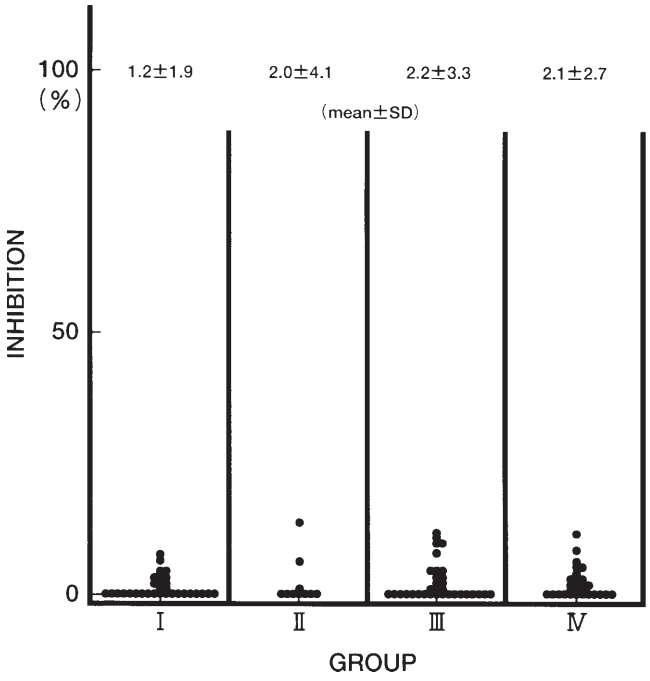

Fig. 4. IgA antibody in human serum against capsular polysaccharide extracted from Smith strain of *Staphylococcus aureus* by enzyme-linked immunosorbent assay inhibition test

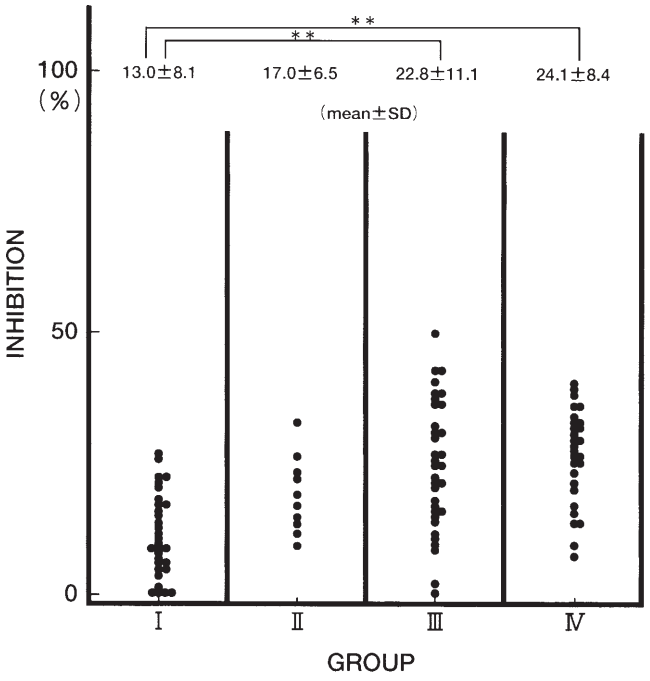

Fig. 6. IgM antibody in human serum against capsular polysaccharide extracted from Reynolds strain of *Staphylococcus aureus* by enzyme-linked immunosorbent assay inhibition test. $^{**}P < 0.01$

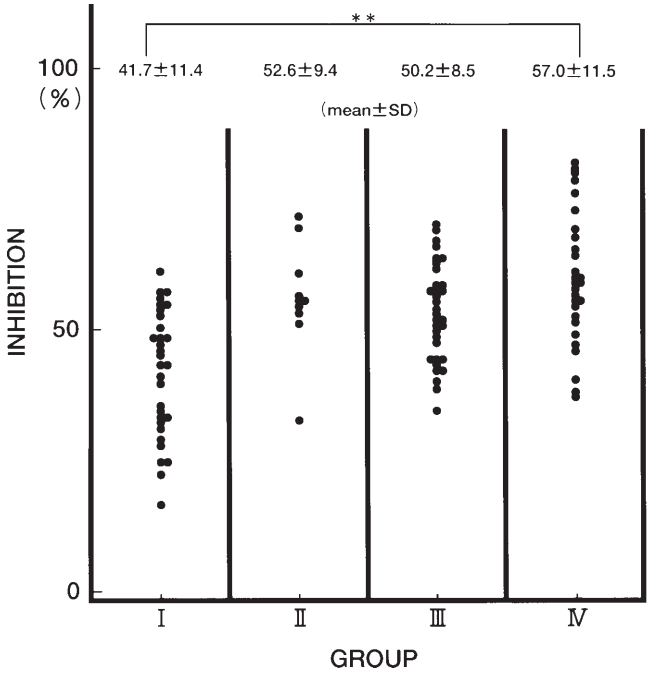

Fig. 5. IgG antibody in human serum against capsular polysaccharide extracted from Reynolds strain of *Staphylococcus aureus* by enzyme-linked immunosorbent assay inhibition test. $^{**}P < 0.01$

IgA. The mean inhibition rates for IgA were 2.0 ± 2.8% in group I, 2.0 ± 4.1% in group II, 3.4 ± 4.1% in group III, and 3.1 ± 3.9% in group IV. There were no significant differences between any of the groups (Fig. 7).

Mean inhibition rates of antibody activity for Becker strain of S. aureus *in human serum*

IgG. The mean inhibition rates for IgG were 32.0 ± 10.6% in group I, 35.5 ± 13.9% in group II, 45.4 ± 12.3% in group III, and 50.0 ± 14.0% in group IV. The values were higher in groups III and IV than in group I. There were significant differences between groups I and III and between groups I and IV ($P < 0.01$), as well as between groups II and IV ($P < 0.05$) (Fig. 8).

IgM. The mean inhibition rates for IgM were 12.5 ± 8.9% in group I, 22.4 ± 8.8% in group II, 24.9 ± 11.2% in group III, and 23.3 ± 9.3% in group IV. The values were higher in groups II, III, and IV than in group I. There were significant differences between groups I and III and between groups I and IV ($P < 0.01$) (Fig. 9), as for IgG values.

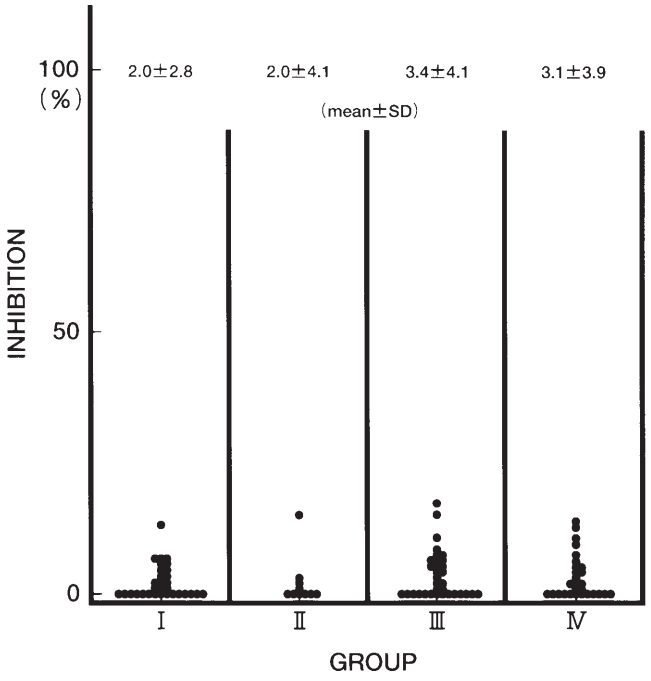

Fig. 7. IgA antibody in human serum against capsular polysaccharide extracted from Reynolds strain of *Staphylococcus aureus* by enzyme-linked immunosorbent assay inhibition test

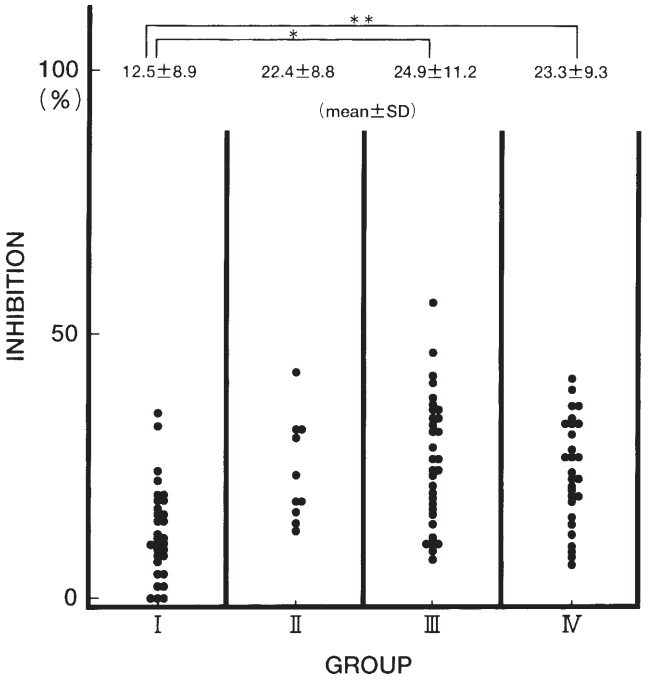

Fig. 9. IgM antibody in human serum against capuslar polysaccharide extracted from Becker strain of *Staphylococcus aureus* by enzyme-linked immunosorbent assay inhibition test. $*P < 0.05$; $**P < 0.01$

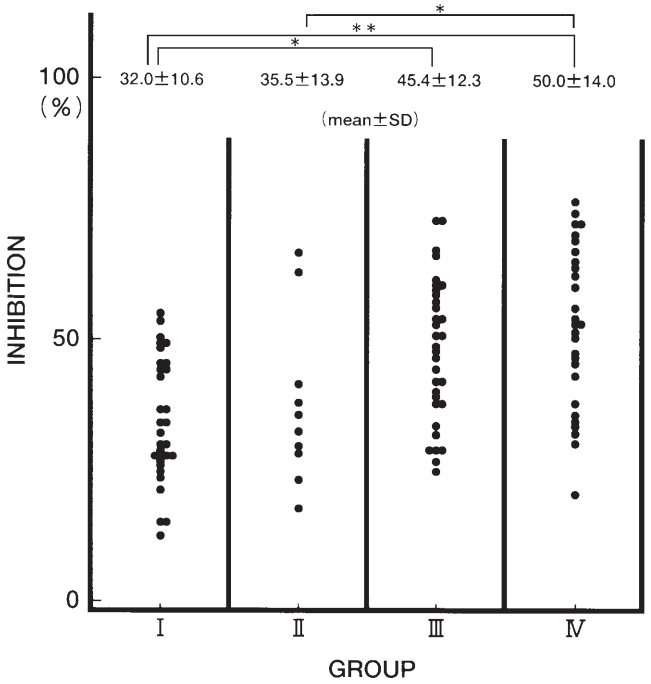

Fig. 8. IgG antibody in human serum against capsular polysaccharide extracted from Becker strain of *Staphylococcus aureus* by enzyme-linked immunosorbent assay inhibition test. $*P < 0.05$; $**P < 0.01$

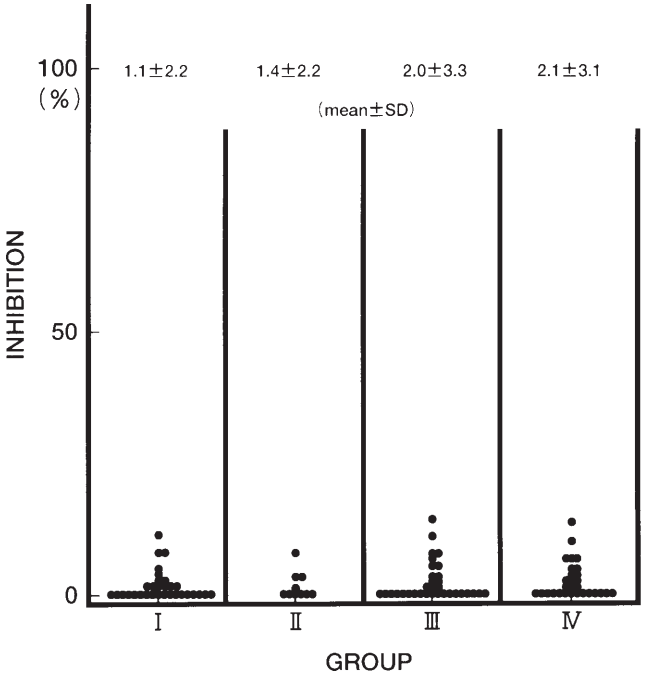

Fig. 10. IgA antibody in human serum against capsular polysaccharide extracted from Becker strain of *Staphylococcus aureus* by enzyme-linked immunosorbent assay inhibition test

IgA. The mean inhibition rates for IgA were 1.1 ± 2.2% in group I, 1.4 ± 2.2% in group II, 2.0 ± 3.3% in group III, and 2.1 ± 3.1% in group IV. There were no significant differences between any of the groups (Fig. 10).

Discussion

Fischer et al.[2] and Yoshida et al.[25] have already reported the presence of protective antibodies against *S. aureus* in normal human serum. Yoshida et al.[25] in particular reported a wide distribution of protective antibodies for mice in normal sera from 100 individuals, using encapsulated strains with different capsular types, confirming that protective antibody against infections is present in normal human serum. However, the importance of bacterial cell surface substances in specific bone marrow lodgement by bacterial was demonstrated in a series of studies of experimental *S. aureus* osteomyelitis.[3,11,14,16] Hirata[4] of our department extracted immunoglobulins specific for the Smith strain from pooled human sera by the method of Yoshida et al.[25] and Yoshida and Ichiman,[26] and reported delayed onset of infection in mice with hematogenous osteomyelitis caused by *S. aureus*. Here, we confirmed the presence of protective antibodies against encapsulated strains of *S. aureus*.

In the present study, we assayed antibody activity for three encapsulated strains representative of capsular types found in clinical isolates.[15] The subjects were divided into three groups: group I, in which *S. aureus* was detected, group II, in which bacteria other than *S. aureus* were detected, group III, bacteria-negative; and group IV, healthy controls. The purpose of this study was to compare differences in antibody activity among the groups by the ELISA inhibition test of Ichiman et al.[7]

In the human serum samples from 102 individuals, the ELISA inhibition rates for the Smith strain (capsular type 2) of *S. aureus* showed antibody activity in the order of group IV, group III, group II, and group I for IgG, (i.e., in the serum of healthy controls, the antibody activity was 2.7 times that in the *S. aureus* detection group). Antibody activity for IgM was also in the order of group IV, group III, group II and group I, with groups III and IV showing antibody activity more than threefold higher than that in group I. These findings agree with reports indicating the importance of IgM as the protective antibody against the Smith strain.[5,6,8,24–26,28] Large amounts of this antibody appear to delay the onset of infections. However, IgA showed no marked differences among our groups, which appears to support the finding that IgA does not exhibit protective effects against infections, since it has no complement-fixing ability.[23] The antigen used in the ELISA inhibition test was the capsular polysaccharide of the Smith strain, and the effective substance used was the Smith surface antigen (SSA), a 2-amino-2-deoxy-D-glucuronic acid conjugate. It has been confirmed that SSA is the only antigen of *S. aureus* strains which protects against infections.[8,24] Since a protective antibody against infections by the Smith strain has been reported[25] in normal human serum in a wide range of experiments in mice,[8,24] it is assumed that the presence of IgM and IgG antibodies for SSA is important for in vivo defense.

When the ELISA inhibition rate in human serum for capsular type 5 and 8 strains (often isolated clinically in recent years)[9,15] was examined, IgG for types 5 and 8 in the controls showed an activity 1.37 times and 1.56 times, respectively, that in the group in which *S. aureus* was detected. IgM activity for types 5 and 8 in the controls was 1.85 and 1.92 times that in the *S. aureus* group, not as marked as the significant difference seen with the Smith strain. IgA showed no marked differences among these strains. At present, both the capsular type 5 and 8 strains are isolated clinically at high rates, and since many of these strains are MRSA,[15] these antibodies will be important in the future even though there is little difference in antibody activity among them. However, the reason for the slight difference between the *S. aureus* group and the control group in antibody activity against the capsular type 5 and 8 strains appeared to be because the pathogenicity of this strain is lower and the capsule is smaller compared with that in the Smith strain. However, the level of antibody activity for weakly pathogenic strains appears to be beneficial for in vivo defense. There are still many unclear aspects of the pathogenicity of this strain, and further studies are required in the future.

The concept that IgG plays the main role in humoral antibodies which protect against infection is still held, and only IgG preparations are used in actual clinical practice. However, treatment with high doses of the immunoglobulin preparation Venoglobulin-I (Green Cross, Osaka, Japan), which contains only IgG, was ineffective in mice infected with *S. aureus*.[8,27,28] Hirata[4] showed the importance of immunoglobulin preparations containing specific IgM in the treatment of experimental osteomyelitis. Vy'mola et al.[18] and Wheat et al.[21,22] reported that IgM antibody served as a infection defense factor against osteomyelitis caused by *S. aureus*. The results of the present study showed that the activity not only of IgG but also of IgM was high in the healthy controls and the bacteria-negative group. The importance of IgM antibody for the capsular antigen in each bacteria group was thus suggested, i.e., it is assumed that the IgM antibody in normal human serum plays an important role as an in vivo defense factor in bone and joint infections by *S. aureus*. This idea is supported by

findings that immunoglobulin preparations containing IgG alone have little effect clinically.

Conclusions

We investigated the presence of protective antibodies against infections by encapsulated *S. aureus* in the serum of patients and healthy individuals as one means to clarify the in vivo defense mechanism in human serum.

The ELISA inhibition assay showed marked inhibition with the capsular type 2, 5, and 8 polysaccharides against 33 serum samples from a non-infected patient group and 28 serum samples from healthy controls, whereas low inhibition was observed with sera from patients with staphylococcal infections. Positive IgG and IgM titers were markedly inhibited with this assay, but no inhibition of IgA titer was obtained in any samples.

These results indicate that, the quantitation of human serum antibody against *S. aureus* capsular polysaccharide by the ELISA inhibition assay demonstrated the protective antibody activities against *S. aureus*.

Acknowledgments. We wish to thank Professor Haruhito Aoki, head of the Department of Orthopedic Surgery, St. Marianna University School of Medicine, for his guidance and for reading the manuscript. We also wish to express our appreciation to Professor Jingoro Shimada, head of the Department of Microbiology, St. Marianna University School of Medicine, for his guidance. Our thanks also go to the staff of the Departments of Orthopedic Surgery and Microbiology, St. Marianna University School of Medicine, for their cooperation in this study.

References

1. Dombrowski ET, Dunn AW. Treatment of osteomyelitis by debridement and closed wound irrigation-suction. Clin Orthop 1965;43:215–31.
2. Fischer NW, Devlin HB, Erlandson AL. A new staphylococcal antigen. Its preparation and immunizing activity against experimental infections. Nature 1963;199:1074–5.
3. Hamabe M. Experimental haematogenous osteomyelitis. Inhibition of bone marrow lodgement in mice by cell surface substance of a stain of *Staphylococcus aureus* (in Japanese). The St. Marianna Med J 1986;14:108–16.
4. Hirata H. Protective effect of specific human immunoglobulins on haematogenous osteomyelitis by *Staphylococcus aureus* in mice (in Japanese). J Jpn Orthop Assoc 1989;63:1545–51.
5. Ichiman Y, Yoshida K. Cross protection in mice with the Smith diffuse strain of *Staphylococcus aureus* versus a type I a strain of group B streptococci. Can J Microbiol 1982;28:726–32.
6. Ichiman Y, Ohshima Y, Yoshida K. Effect of specific protective immunoglobulins extracted from pooled human sera on the treatment of mice with lethal infection of methicillin-resistant *Staphylococcus aureus*. Recent Adv Chemo 1985;1:797–8.
7. Ichiman Y, Suganuma M, Takahashi M, et al. Relation of human serum antibody against *Staphylococcus epidermidis* cell surface polysaccharide detected by enzyme-linked immunosorbent assay to passive protection in the mouse. J Appl Bacteriol 1991;71:176–81.
8. Ichiman Y. The role of capsule in staphylococcal infection and immunity (in Japanese). Jpn J Bacteriol 1996;51:601–11.
9. Karakawa WW, Fournier JM, Vann WF, et al. Method for the serological typing of the capsular polysaccharides of *Staphylococcus aureus*. J Clin Microbiol 1985;22:445–7.
10. Kawashima M, Iwabuchi R, Torisu T. Antibiotic therapy for osteomyelitis (in Japanese). Orthop Surg Traumatol 1979;22:381–6.
11. Matsushita K, Hamabe M, Matsuoka M, et al. Experimental hematogenous osteomyelitis by *Staphylococcus aureus*. Clin Orthop 1997;334:291–7.
12. Miyoshi S. Studies on the treatment of chronic pyogenic osteomyelitis — effect of improved continuous closed irrigation method (in Japanese). J Jpn Orthop Assoc 1969;43:285–98.
13. Morse SI. Isolation and properties of surface antigen of *Staphylococcus aureus*. J Exp Med 1962;155:295–311.
14. Shoji I. Histological studies on experimental haematogenous osteo-myelitis by a strain of *Staphylococcus aureus* in hyperimmune mice (in Japanese). The St. Marianna Med J 1989;17:744–52.
15. Sompolinsky D, Samra Z, Karakawa WW, et al. Encapsulation and capsular types in isolates of *Staphylococcus aureus* from different sources and relationship to phage types. J Clin Microbiol 1985;22:828–34.
16. Takeda H. Experimental hematogenous osteomyelitis: Development of bone marrow lodgement of a strain of *Staphylococcus aureus* in hyperimmune mice (inJapanese). The St. Marianna Med J 1987;15:90–9.
17. Uden A, Danielsson LG. The diagnosis and treatment of acute haematogenous osteomyelitis. Acta Orthop Scand 1980; 51:384.
18. V'ymola F, Lochman O, Buda J, et al. Staphylococcal osteomyelitis. Ann NY Acad Sci 1974;236:508–14.
19. Waldvogel FA, Medoff G, Swartz MN. Osteomyelitis: A review of clinical features, therapeutic considerations and unusual aspects (First of three parts). N Engl J Med 1970;5:198–206.
20. Waldvogel FA, Vasey H. Osteomyelitis. The past decade. N Engl J Med 1980;303:360–70.
21. Wheat LJ, Kohler RB, Tabbarah Z A, et al. IgM antibody response to staphylococcal infection. J Infect D is 1981;144:307–11.
22. Wheat LJ, White AC, Norden C. Serological diagnosis of *Staphylococcus aureus* osteomyelitis. J Clin Microbiol 1985;21: 764–7.
23. Wilfert CM. *Neisseria meningitis*. In: Joklik WK, Willett HP, Amos DB, editors. Zinsser microbiology. 18th edition. Norwalk Conneticut: Appleton-Century-Crofts, 1984:493–8.
24. Yoshida K, Ekstedt RD. Antibody response to *Staphylococcus aureus* in rabbits: Sequence of immunoglobulin synthesis and its correlation with passive protection in mice. J Bacteriol 1968;96:1540–5.
25. Yoshida K, Ichiman Y, Narikawa S, et al. Passive protection by human serum in mice infected with encapsulated *Staphylococcus aureus*. J Med Microbiol 1979;12:277–282.
26. Yoshida K, Ichiman Y. Successive extraction of specific protective immunoglobulins from pooled human sera. J Clin Microbiol 1984;20:461–4.
27. Yoshida K, Ichiman Y, Ohshima Y, et al. Serological properties of specific immunoglobulin against Smith strain of *Staphylococcus aureus* extracted from human sera (in Japanese). Jpn J Infect Dis 1985;59:433–8.
28. Yoshida K, Ichiman Y, Ohshima Y. Human specific immunoglobulin protects against infection with common *Staphylococcus* in mice. Experientia 1987;43:329–30.