Therapeutic Effect of Modified Oligopeptides from the Beetle *Allomyrina dichotoma* on Methicillin-resistant *Staphylococcus aureus* (MRSA) Infection in Mice

Manabu YAMADA1), Kikuyasu NAKAMURA1), Hisako SAIDO-SAKANAKA2), Ai ASAOKA2).

Minoru YAMAKAWA2,3), Yu YAMAMOTO3), Ayako KOYAMA3), Kenji HIROSUKA3), Akira SHIMIZU4) and Yoshikazu HIROTA1,5)

1)National Institute of Animal Health, Tsukuba, Ibaraki 305–0856, 2)National Institute of Agrobiological Sciences, Tsukuba, Ibaraki 305–8634, 3)University of Tsukuba, Tsukuba, Ibaraki 305–8572, Japan, 4)University of Kobe, Kobe, Hyogo 657–8501 and 5)Tokyo University of Agriculture and Technology, Fuchu, Tokyo 183–8509, Japan

(Received 11 April 2005/Accepted 16 June 2005)

**ABSTRACT.** Anti-bacterial activity of two synthesized oligopeptides, RLYLRIGRR-NH2 (peptide A) and RLRLRIGRR-NH2 (peptide B), both which based on a putative active site of defenses, an anti-bacterial peptide from the beetle *Allomyrina dichotoma*, was examined by macroscopic and histopathologic assessment during the course of infection in mice inoculated with methicillin-resistant *Staphylococcus aureus* (MRSA) in vivo. Both peptides A and B decreased the mortality of mice inoculated with MRSA. Peptides A and B decreased the macroscopic and histopathologic lesions by MRSA infection in mice even seven days after the challenge. The anti-bacterial activity of peptides A and B has a therapeutic effect on MRSA infection in mice even seven days after being challenged.

**KEY WORDS:** *Allomyrina dichotoma*, defensin analogue, histopathology, methicillin-resistant *Staphylococcus aureus* (MRSA), mouse.

The rapid spread of antibiotic-resistant pathogenic bacteria in hospitals causes severe problems [2, 8]. The search for a new anti-bacterial agent is crucial for controlling antibiotic-resistant pathogenic bacteria [2, 8]. Insect anti-bacterial peptides have unique properties that disrupt bacterial membranes via peptide-lipid interaction [1], and some are known to be effective against antibiotic-resistant pathogenic bacteria [5, 9, 13]. Insect anti-bacterial peptides are generally active against gram-positive but not gram-negative bacteria [6].

We previously designed and synthesized oligopeptides based on the amino-acid sequence of *Allomyrina dichotoma* (A. dichotoma) defensin [11]. These oligopeptides (8- to 12-mer peptides) were effective against both Gram-positive and negative bacteria [11]. Moreover, we modified a putative active site of *A. dichotoma* defensin and synthesized some novel peptides to increase anti-bacterial activity [10]. These synthetic oligopeptides possessed stronger anti-bacterial activity and interacted with liposomal membranes of *Staphylococcus aureus* (S. aureus) and *Escherichia coli* (E. coli) [10]. Furthermore, some of these synthetic oligopeptides demonstrated stronger anti-bacterial activity even against antibiotic-resistant pathogenic bacteria in the *in vitro* study [10].

There have been many reports of *in vitro* studies concerning the anti-bacterial activities of insect anti-bacterial peptides [5, 9, 13], however, very few studies using experimental animals have been described [13]. We previously reported the macroscopic and histopathologic effects of two newly modified oligopeptides, RLYLRIGRR-NH2 (peptide A) and RLRLRIGRR-NH2 (peptide B), from the beetle *A. dichotoma* on antibiotic-resistant *E. coli* infection in mice [14]. The results of macroscopic and histopathologic examinations revealed that peptide B could decrease lesions from antibiotic-resistant pathogenic *E. coli* infection in the mice [14]. In contrast, peptide A failed to protect mice from infection with antibiotic-resistant pathogenic *E. coli* [14]. This paper describes the therapeutic effect of modified oligopeptides from the beetle *A. dichotoma* on MRSA infection in mice.

**MATERIALS AND METHODS**

**Peptide:** The two oligopeptides, RLYLRIGRR-NH2 (peptide A) and RLRLRIGRR-NH2 (peptide B), used in this study were designed and synthesized using methods from our previous studies [10, 11].

**Mice:** Four-week-old male C57BL/6J Jcl mice were purchased from Nippon Clea (Tokyo, Japan). The animals received a common laboratory diet and water *ad libitum* and were kept at a constant temperature (22°C) in a negatively pressurized animal house in filtered air. This study was performed according to the Guidelines for Animal Experiments of the National Institute of Animal Health, Japan. All mice had been immunsuppressed by pretreatment with daily intramuscular injections of dexamethasone (DM) (Riken Chikusan Kayaku, Tokyo) at a dose of 0.1 mg/kg bodyweight for five days before inoculation.

**Bacteria:** The No. 8 strain of MRSA used in this study was isolated from the naris of a dog with otitis exterma. The isolated MRSA was characterized by the production of type II coagulase, enterotoxin C, and toxic shock syndrome toxin-1 (TSST-1) and showed as antimicrobial resistance pattern of oxacillin (MIPIC)/ cefazolin (CZ) imipenem (IPM)/ erythromycin (EM)/ tetracycline (TC)/ minocycline (MINO)/ kanamycin (KM)/ norfloxacin (NFLX)/ lincomyci-
cin (LCM). MRSA was grown in heart-infusion broth for 20 hr at 37°C, and the viable numbers were adjusted to those required for inoculation. All mice inoculated intraperitoneally with $4 \times 10^8$ CFU/mouse of this strain in the pilot examinations died within seven days. No mice inoculated intraperitoneally with $1 \times 10^7$ CFU/mouse died within three weeks. Intraperitoneal abscesses in the mice inoculated intraperitoneally with $1 \times 10^7$ CFU/mouse died within three weeks. Continuous swelling and congestion of the penis were observed between days 15 and 20 after the challenge in the pilot examinations. Those lesions appeared to be due to the pathogenicity of MRSA in mice (unpublished data).

Experiment 1: The experimental groups of mice are summarized in Table 1. Groups 1 to 3 were inoculated intraperitoneally with $4 \times 10^8$ CFU/mouse. Group 2 was injected intraperitoneally with 0.5 mg/mouse peptide A immediately after inoculation of MRSA. Group 3 was injected intraperitoneally with 0.5 mg/mouse peptide B immediately after inoculation of MRSA. Group 1 was infected control group. Groups 4 to 6 were non-infected control groups. Group 4 was a negative control group injected only the medium and buffered saline, group 5 with peptide A alone, and group 6 with peptide B alone. All surviving mice were killed with diethyl ether, perfused with 10% buffered formalin through the heart, and immediately necropsied at seven days after inoculation of MRSA. All abdominal and thoracic organs, particularly the urinary organs, including the penis, were carefully observed at necropsy for macroscopic findings.

Statistics: The mortality rates of mice and a comparison of the lesions of peptides-treated and untreated mice challenged with MRSA were analyzed using Fisher’s exact test.

Histopathology: All abdominal and thoracic organs, including the liver, kidney and penis, were collected for histopathological examination. The tissue samples were fixed in 10% buffered formalin, embedded in paraffin wax, and sectioned at 4 µm. Dewaxed sections were stained with hematoxylin and eosin (HE) and Gram stain.

RESULTS

Experiment 1

Mortality: Table 1 shows the mortality rates. The infected control group (group 1) exhibited high mortality rates (85%). In contrast, the mortality rates in the ether peptide A or B injected group (groups 2 and 3) were significantly reduced compared to the infected control group. In the non-infected control groups (groups 4 to 6) all mortality rates were 0%. Within seven days, no apparent changes in the physical, social, or feeding activities were observed in the mice with peptide alone.

Macroscopic findings: In the infected control group inoculation of MRSA. At necropsy, all abdominal and thoracic organs, especially liver and kidney, were carefully observed for macroscopic findings.

Experiment 2: The experimental groups of mice are summarized in Table 2. Groups 7 to 9 were inoculated intraperitoneally with $1 \times 10^7$ CFU/mouse. Group 8 was injected intraperitoneally with 0.5 mg/mouse peptide A seven days after inoculation of MRSA. Group 9 was injected intraperitoneally with 0.5 mg/mouse peptide B seven days after inoculation of MRSA. Group 7 was the infected control group. Groups 10 to 12 were non-infected control groups. Group 10 was a negative control group injected only the medium and buffered saline, group 11 was treated with peptide A alone, and group 12 with peptide B alone. All surviving mice were killed with diethyl ether, perfused with 10% buffered formalin through the heart, and immediately necropsied 21 days after inoculation of MRSA. All abdominal and thoracic organs, particularly the urinary organs, including the penis, were carefully observed at necropsy for macroscopic findings.

Table 1. The mortality rates of examined groups within seven days (experiment 1)

<table>
<thead>
<tr>
<th>Group</th>
<th>MRSA Dose (cfu)</th>
<th>Injection of peptide</th>
<th>Mortality Rate (dead/n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$4 \times 10^8$</td>
<td>–</td>
<td>85% (34/40)</td>
</tr>
<tr>
<td>2</td>
<td>$4 \times 10^8$</td>
<td>A</td>
<td>22.5% (9/40)*</td>
</tr>
<tr>
<td>3</td>
<td>$4 \times 10^8$</td>
<td>B</td>
<td>32.5% (13/40)*</td>
</tr>
<tr>
<td>4</td>
<td>–</td>
<td>–</td>
<td>0% (0/10)</td>
</tr>
<tr>
<td>5</td>
<td>–</td>
<td>A</td>
<td>0% (0/10)</td>
</tr>
<tr>
<td>6</td>
<td>–</td>
<td>B</td>
<td>0% (0/10)</td>
</tr>
</tbody>
</table>

A: RLYLRIGRR-NH$_2$; B: RLRLRIGRR-NH$_2$; -: Injection with only the medium or buffered saline; *: p<0.001 compared with the infectious control group (group 1).

Table 2. Comparison of the macroscopical appearance in mice 21 days after challenge (experiment 2)

<table>
<thead>
<tr>
<th>Group</th>
<th>MRSA Dose (cfu)</th>
<th>Injection of peptide</th>
<th>Penial lesion (affected/n)</th>
<th>Subcutaneous nodular lesion (affected/n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>$1 \times 10^7$</td>
<td>–</td>
<td>72.5% (29/40)</td>
<td>92.5% (37/40)</td>
</tr>
<tr>
<td>8</td>
<td>$1 \times 10^7$</td>
<td>A</td>
<td>0% (0/40)*</td>
<td>0% (0/40)*</td>
</tr>
<tr>
<td>9</td>
<td>$1 \times 10^7$</td>
<td>B</td>
<td>0% (0/40) *</td>
<td>30% (12/40)*</td>
</tr>
<tr>
<td>10</td>
<td>–</td>
<td>–</td>
<td>0% (0/10)</td>
<td>0% (0/10)</td>
</tr>
<tr>
<td>11</td>
<td>–</td>
<td>A</td>
<td>0% (0/10)</td>
<td>0% (0/10)</td>
</tr>
<tr>
<td>12</td>
<td>–</td>
<td>B</td>
<td>0% (0/10)</td>
<td>0% (0/10)</td>
</tr>
</tbody>
</table>

A: RLYLRIGRR-NH$_2$; B: RLRLRIGRR-NH$_2$; -: Injection with only the medium or buffered saline; *: p<0.001 compared with the infectious control group (group 7).
(group 1), the macroscopic appearance of the liver and kidney changed significantly compared to the non-infected control groups (groups 4 to 6) (date not shown). Multiple white nodules were observed in the surface of the liver, kidney, pancreas, spleen, diaphragm, and peritoneum (Fig. 1a). The kidney was pale in color. The cut surface of the kidney showed a mosaic appearance of irregularly shaped red, gray, and white areas (Fig. 2a). The thymus was atrophic due to DM treatment.

There were no nodular lesions in the infected mice with either peptide A or B injection (groups 2 and 3). The liver and kidney of group 2 were not swollen, and the surface and cut surface appeared almost normal (Figs. 1b, 2b). In the peptide B-injected group (group 3), the surface of the kidney was pale in color but no nodular lesions were seen (Fig. 1c). In the cut surface of kidney of group 3, slight changes appeared as white areas were occasionally seen (Fig. 2c). The peptide A and B provided a useful effect on a macroscopic protection in mice infected with MRSA. There was no macroscopic change in the examined organs of non-infected groups (groups 4 to 6).

Histopathological findings: In the infected control group (groups 1), large encapsulated abscesses were seen in the subserosal part of the liver, kidney, pancreas, spleen, diaphragm and peritoneum. Significantly large numbers and a large area of necrosis were found in the liver and kidney (Figs. 3a, 4a). In the necrotic foci, gram-positive bacterial colonies were frequently observed (Fig. 5a).

In the peptide A and B injection groups (groups 2 and 3), the necrotic foci appeared small and the numbers of necrotic foci were significantly reduced (Figs. 3b, 3c, 4b, 4c). Hepatic cords were almost normally organized. Large abscess was not observed. The lesions were significantly fewer than in the infected control groups (date not shown). No bacterial colonies were observed in the necrotic foci. In the high magnification of the Gram stained section, a few gram-positive bacteria was only seen in the lesions (Figs. 5b, 5c). The peptide A and B taken in a single dose of 0.5 mg achieved a significant overall reduction in histopathological lesions. There were no histopathological changes in the examined organs of non-infected control groups (groups 4 to 6).

Experiment 2

Macroscopic findings: The mortality rate of all groups was 0%. The macroscopic findings of the mice injected with either peptide A (group 8) or B (group 9) were significantly fewer than in the infected control group (group 7) (Table 2). Severe swelling and congestion of the penis were observed in group 1 (Fig. 6a). Subcutaneous nodular lesions were seen at the hypogastrum (Fig. 6a). Multiple white nodules were observed on the surface of the liver, kidney, pancreas, spleen, diaphragm, and peritoneum. The kidney was pale in color. The cut surface of the kidney exhibited a mosaic appearance of irregularly shaped red, gray, and white areas. The ureter was significantly swollen. The wall of the bladder was thickened and cloudy.

There were no subcutaneous or penile lesions in the peptide A injected group (group 8) (Fig. 6b, Table 2). Subcutaneous nodules appeared small in the peptide B injected group (group 9) (Fig. 6c), and the incidence was reduced compared to the infected control group (Table 2). No penile lesions were observed in group 9 (Fig. 6c). Slight lesions were seen as only pin-spot-sized white spots on the surface of the organs of mice injected with either peptide A or B seven days after infection (groups 8 and 9). Slight changes appearing as white areas were occasionally seen in the cut surface of the kidneys of peptide-injected mice (groups 8 and 9). The ureter and bladder exhibited normal appearance. Peptides A and B provided a useful effect in macroscopic protection of mice infected with MRSA. There were no macroscopic changes in the examined organs of the non-infected groups (groups 10 to 12).

Histopathological findings: There were significantly fewer lesions caused by MRSA in the peptide A- and B-injected groups (groups 8 and 9) than in the infected control group (group 7) (date not shown). Large encapsulated abscesses were seen in the subserosal parts of the liver, kidney, pancreas, spleen, diaphragm, and peritoneum in the infected control group (group 7). Many necrotic foci were found in the liver and kidney (Fig. 7a). Gram-positive bacterial colonies were observed in the necrotic foci (Fig. 8a). The renal pelvis was dilated with cell debris, neutrophiles, pus, and bacteria. Severe ureteritis, cystitis, and urethritis were observed in the urinary tract. Purulent lesions in the penis involved the urethra, which was severely dilated with cell debris, neutrophiles and pus (Fig. 9a).

There were no purulent lesions in the examined organs in the peptide A-injected group (group 8). Multifocal organization caused by fibrosis was observed in the kidney (Fig. 7b). No bacterial colonies or abscesses were observed (Fig. 7b). The wall of the ureter was somewhat thickened. Only slight infiltration of neutrophiles and lymphocytes were seen in the bladder and urethra. No Gram-positive bacterial colonies were observed (Fig. 8b). No histological changes were observed in the penis, such as congestion, edema, or purulent lesions (Fig. 9b).

Subcutaneous abscesses were observed in the peptide B-injected group (group 9). The necrotic foci appeared small and were organized by fibrogranulomatous tissue. No bacterial colonies were observed. In the kidney, multifocal organization caused by fibrosis was observed, but no purulent lesions (Fig. 7c). The lesions in the urinary tract were as minimal as in group 8. A few Gram-positive bacterial colonies were observed (Fig. 8c). The penis presented an almost normal appearance (Fig. 9c). Peptides A and B taken in a single dose of 0.5 mg achieved a significant overall reduction in histopathological lesions. There were no histopathological changes in the examined organs of the non-infected control groups (groups 10 to 12).

DISCUSSION

In this study, both peptide A and B decreased mortality rates of mice inoculated with MRSA. The results of macro-
Figs. 1 and 2. Effect of peptides on macroscopical appearance of the formalin fixed kidney. 1a. Seven days after inoculation with $4 \times 10^8$ CFU of MRSA. In the surface of the kidney, nodular masses are seen. 1b. Seven days after inoculation with $4 \times 10^8$ CFU of MRSA and with injection of peptide A. The surface appears almost normal. 1c. Seven days after inoculation with $4 \times 10^8$ CFU of MRSA and with injection of peptide B. The surface show pale in color, but no nodular masses. 2a. Seven days after inoculation with $4 \times 10^8$ CFU of MRSA. The cut surface of the kidney shows a mosaic appearance of irregularly shaped red, gray and white areas. 2b. Seven days after inoculation with $4 \times 10^8$ CFU of MRSA and with injection of peptide A. The cut surface appears almost normal. 2c. Seven days after inoculation with $4 \times 10^8$ CFU of MRSA and with injection of peptide B. The surface show slight changes appeared as white areas.
scopical and histopathologic examinations of mice revealed that peptides A and B possess anti-bacterial activity against pathogenic MRSA. The urinary tract, including the kidneys, was most severely affected by pathogenic MRSA in this study. The lesions in the kidneys were progressive and seemed to develop in the ureter and bladder via descent from the penis. Purulent lesions in the kidney were healed by fibrosis and absorption in the groups injected with the peptide seven days after the challenge, leaving a scar. The progression of lesions to the ureter and bladder via the penis was inhibited. This result suggests that the anti-bacterial activity of peptides A and B against MRSA was effective even seven days after the challenge.

A report of an anti-bacterial agent based on the cyclic D,L-α-peptide architecture studied the in vivo anti-bacterial efficacy of peptide nanotubes, as determined by the survival rates of treated versus untreated mice challenged with lethal doses of MRSA [4]. We designed an in vivo assessment of the anti-bacterial efficacy of insect oligopeptides using macroscopic and histopathologic assays. While assessment of anti-bacterial activity of insect oligopeptides using an in vitro assay or biological methods has been widely examined [5, 9, 10, 11, 13], there are no reports of in vivo macroscopic or histopathologic assessment using experimental animals, except for our previous study using mice infected with anti-biotic-resistant pathogenic E. coli [14]. The therapeutic effect of peptides against MRSA infection in mice was examined in this study when the peptides were injected seven days after the challenge; the previous study examined the anti-bacterial activity when the agents were injected at almost the same time as the challenge [10, 14]. Peptide A displayed anti-bacterial activity against MRSA but not against antibiotic-resistant pathogenic E. coli in our consecutive in vivo studies [14]. In contrast, peptide B demonstrated anti-bacterial activity against both MRSA and antibiotic-resistant pathogenic E. coli [14]. Insect defensins are generally active against Gram-positive but not Gram-negative bacteria [6]. Our peptide B possesses a wide range of anti-bacterial activity against both gram-negative and gram-positive organisms resistant to antibiotic agents. Peptide A displayed greater anti-bacterial activity against Gram-positive bacteria, such as S. aureus, than against Gram-negative bacteria, such as E. coli [10]. Conversely, peptide B tended to demonstrate greater anti-bacterial activity against Gram-negative bacteria than against Gram-positive bacteria in the in vitro study [10]. Peptide A tended to be more active against MRSA than peptide B in this study, same as the previous in vitro study. The differences in the physiological functions of the membranes between gram-positive bacteria and gram-negative bacteria may be related, leading to the difference in the anti-bacterial activity of these peptides.

The anti-bacterial effect of modified peptide analogues was due to their interaction with bacterial membranes, judging from the leakage of liposome-entrapped glucose [3, 11]. The present results showed that the numbers of gram-positive bacterial colonies in the lesions were significantly reduced by injection of the peptides. The precise mechanisms of the anti-bacterial effects of the peptides in the intra-peritoneal environment of mice remains to be clarified; it is possible that peptides inhibit the growth of bacteria due to interaction with bacterial membranes, as in the in vitro study. On the other hand, our two peptides suppressed tumor necrosis factor-alpha (TNF-α) gene expression induced by lipoteichoic acid (LTA) in murine macrophages [10]. TNF-α plays an important rule in the control of S. aureus infection and regulates the ensuing host immune response [7, 12]. Suppression of TNF- gene expression may also contribute to the recovery of mice from Gram-positive bacterial sepsis. We believe that a strategy to modify insect anti-bacterial peptides is an important approach to developing novel antibiotics. A. dichotoma oligopeptides can serve as lead peptides in developing antibiotics effective against infectious diseases caused by drug-resistant bacteria in hospitals.

**ACKNOWLEDGEMENTS.** This work was supported by a Grant-in-Aid (Promotion of Basic Research Activities for Innovative Biosciences) from the Bio-oriented Technology Research Advancement Institution, Japan. We thank Mr. M. Kobayashi and Miss M. Shimada for preparing the histological tissue sections and Dr. Y. Ando and Mr. T. Fujisawa for preparing the photographs.
REFERENCES


Fig. 6. Effect of peptides on macroscopical appearance of the mice. 6a. 21 days after inoculation with 1 × 10^7 CFU of MRSA. Swelling and congestion of the penis is severely seen (white arrow). Subcutaneous nodular lesion is also seen at the hypogastrium (black arrow). 6b. 21 days after inoculation with 1 × 10^7 CFU of MRSA and with 14 days after injection of peptide A. No subcutaneous and penial lesions. 6c. Seven days after inoculation with 1 × 10^7 CFU of MRSA and with 14 days after injection of peptide B. Subcutaneous nodules appeared small (black arrow). Penial lesions is not seen. The lesions are significantly fewer than in the infected control group.

Figs. 7 and 8. Effect of peptides on histopathological finding of the kidney. 7a. 21 days after inoculation with 1 × 10^7 CFU of MRSA. A large area of necrosis with bacterial colonies is seen. HE stain. Bar = 800 µm. 7b. 21 days after inoculation with 1 × 10^7 CFU of MRSA and with 14 days after injection of peptide A. The purulent lesions in the kidney healed by fibrosis leaving a scar. The lesions are significantly fewer than in the infected control group. HE stain. Bar = 400 µm. 7c. 21 days after inoculation with 1 × 10^7 CFU of MRSA and with 14 days after injection of peptide B. The lesions are significantly fewer than in the infected control group. HE stain. Bar = 400 µm. 8a. 21 days after inoculation with 1 × 10^7 CFU of MRSA. Gram-positive bacteria are frequently observed. Gram stain. Bar = 40 µm. 8b. 21 days after inoculation with 1 × 10^7 CFU of MRSA and with 14 days after injection of peptide A. No Gram-positive bacteria in the lesion. Gram stain. Bar = 40 µm. 8c. 21 days after inoculation with 1 × 10^7 CFU of MRSA and with 14 days after injection of peptide B. No Gram-positive bacteria in the lesion. Gram stain. Bar = 40 µm.

Fig. 9. Effect of peptides on histopathological finding of the penis. 9a. 21 days after inoculation with 1 × 10^7 CFU of MRSA. Swelling and congestion of the penis is seen. The urethra is severely dilated with cell debris, neutrophilites and pus. HE stain. Bar = 800 µm. 9b. 21 days after inoculation with 1 × 10^7 CFU of MRSA and with 14 days after injection of peptide A. No subcutaneous and penial lesions. HE stain. Bar = 550 µm. 9c. 21 days after inoculation with 1 × 10^7 CFU of MRSA and with 14 days after injection of peptide B. Subcutaneous abscess is seen. Penial lesion is not seen. HE stain. Bar = 800 µm.