Decreased affinity of mosaic-structure recombinant penicillin-binding protein 2 for oral cephalosporins in Neisseria gonorrhoeae

Susumu Ochiai1,2*, Satomi Sekiguchi1, Akio Hayashi1, Mitsunobu Shimadzu1, Hiroaki Ishiko1, Rie Matsushima-Nishiwaki3, Osamu Kozawa3, Mitsuru Yasuda2 and Takashi Deguchi2

1Research and Development Department, Mitsubishi Kagaku Bio-Clinical Laboratories, Inc., 3-30-1 Shimura, Itabashi-Ku, Tokyo 174-8555, Japan; 2Department of Urology, Gifu University School of Medicine, 1-1 Yanagido, Gifu City 501-1194, Japan; 3Department of Pharmacology, Gifu University School of Medicine, 1-1 Yanagido, Gifu City 501-1194, Japan

Received 20 October 2006; returned 7 January 2007; revised 18 April 2007; accepted 22 April 2007

Objectives: In Neisseria gonorrhoeae, the mosaic structure of penicillin-binding protein 2 (PBP 2), composed of fragments of PBP 2 from Neisseria cinerea and Neisseria perflava, was significantly associated with decreased susceptibility to cephalosporins, particularly oral cephalosporins. The aim of this study was to determine the affinity of mosaic PBP 2 for cephalosporins in N. gonorrhoeae.

Methods: Two types of non-mosaic PBP 2 from the type strain of N. gonorrhoeae (ATCC 19424) and a clinical strain (GU01-29), as well as the mosaic PBP 2 from a clinical strain (GU01-89), were expressed in insect cells, and recombinant PBP 2s were purified. ATCC 19424 and GU01-29 were susceptible to cephalosporins. GU01-89 showed decreased susceptibility to cephalosporins. Bindings of fluorescent penicillin to PBP 2 were characterized by the Scatchard plot analysis. The affinity of the recombinant PBP 2s for cefdinir, cefixime and ceftriaxone was determined by PBP 2 competition assays with fluorescent penicillin.

Results: The \( K_d \) value of mosaic PBP 2 for fluorescent penicillin was higher than that of non-mosaic PBP 2s. The affinity of mosaic PBP 2 for cefdinir or cefixime was lower than that of the non-mosaic PBP 2s. The affinity of the mosaic PBP 2 for ceftriaxone was not changed, compared with that of the non-mosaic PBP 2s.

Conclusions: Other mechanisms may be involved in clinical isolates with decreased susceptibility to cephalosporins, but this study suggests that the decreased affinity of mosaic-structure recombinant PBP 2 for oral cephalosporins may contribute to decreased susceptibility to these antibiotics in N. gonorrhoeae.

Keywords: mosaic, PBP 2, cephem, fluorescent penicillin, competition assay

Introduction

Penicillins and tetracyclines were commonly used for the treatment of gonococcal infections until the emergence of penicillin- and tetracycline-resistant strains of Neisseria gonorrhoeae. Fluoroquinolones and broad-spectrum cephalosporins have since been used as primary therapies for uncomplicated gonococcal infections. Since the mid-1990s in Japan, however, the failure of fluoroquinolone treatment, related to the development of fluoroquinolone resistance in N. gonorrhoeae strains, has been reported. Therefore, cephalosporins are now used as the primary treatment for gonococcal infections, although certain clinical isolates of N. gonorrhoeae have been found to be less susceptible to oral cephalosporins, including cefixime, in Japan.

Penicillin-binding proteins (PBPs) are membrane-bound enzymes that catalyse the final steps in cell wall biosynthesis and are the targets of the \( \beta \)-lactam antibiotics. Cephalosporins prevent cell wall synthesis in N. gonorrhoeae by binding to PBPs on the cytoplasmic membrane. Analyses of...
membrane fractions of *N. gonorrhoeae* labelled with radioactive penicillin indicate that this bacterial species possesses three PBPs: PBP 1, 2 and 3.\(^3\)\(^,\)\(^4\)\(^,\)\(^1\) Analysis of the complete genomic sequence of *N. gonorrhoeae* indicates the existence of a fourth PBP, PBP 4.\(^6\) Inhibition of PBP 1 or 2 may result in the inhibition of bacterial growth, whereas PBPs 3 and 4 may not be involved in cell viability.\(^1\)\(^,\)\(^7\) PBPs 1 and 2 are major targets of β-lactam antibiotics used against *N. gonorrhoeae*. Many studies of the molecular mechanisms underlying the resistance of *N. gonorrhoeae* to β-lactam antibiotics have been reported. *penA* causes the insertion of a single amino acid into PBP 2, reducing penicillin binding.\(^1\)\(^8\) Recently, *penA1* was shown to be involved in penicillin resistance.\(^1\)\(^9\) *penA1* encodes PBP 1, containing a single amino acid alteration of Leu-421→Pro, which decreases the rate of acylation with β-lactam antibiotics. *penC* is required to transform an intermediate-level penicillin-resistant strain possessing *penA1* into a high-level resistance strain. In addition, the mechanisms for chromosomally mediated resistance of *N. gonorrhoeae* to penicillin G involve *penB* and *mtr* mutations.\(^2\)\(^0\) The *penB* mutation, which is linked to the porin gene, reduces porin permeability to hydrophobic antibiotics and plays an important role in the development of resistance to penicillin G, cephalosporins and tetracycline. *mtr* increases expression of the *MtrCDE* efflux pump and modulates gonococcal resistance to hydrophobic agents.\(^2\)\(^1\) However, these mechanisms appear not to affect the susceptibilities of *N. gonorrhoeae* strains to cephalosporins so much as to threaten the efficacy of currently recommended cephalosporin regimens for the treatment of gonorrhoea. Recently, the *penA* gene of clinical isolates of *N. gonorrhoeae* with decreased susceptibility to oral cephalosporins in Japan was reported to have a mosaic structure.\(^2\)\(^2\) We also identified a mosaic PBP 2 in clinical isolates of *N. gonorrhoeae*, composed of fragments of PBP 2 from *Neisseria cinerea* and *Neisseria phflava*.\(^2\)\(^3\) This mosaic alteration of PBP 2 showed significant association with decreased susceptibility to cephalosporins. One of the assumed mechanisms underlying the decreased susceptibility to cephalosporins is the reduced affinity of the mosaic PBP 2 for these agents. However, no study on the affinity of mosaic PBP 2 for cephalosporins has yet been reported.

In this study, two types of *N. gonorrhoeae* PBP 2, mosaic and non-mosaic, were expressed in insect cells by means of a baculovirus expression vector system, and a large quantity of recombinant PBP 2 was purified. Bindings of fluorescent penicillin to PBP 2 were characterized by the Scatchard plot analysis. PBP 2 competition assays with fluorescent penicillin were performed to determine the affinity of each of the recombinant PBP 2s for β-lactam antibiotics, including penicillin V, cefdinir, cefixime and ceftriaxone.

### Materials and methods

**N. gonorrhoeae strains**

This study used the type strain of *N. gonorrhoeae* (ATCC 19424) and two clinical strains of *N. gonorrhoeae* (GU01-29 and GU01-89) isolated from men with gonorrhoea at Gifu University Hospital or its affiliated hospital in central Japan. These strains were included in our previous study, in which susceptibilities to penicillin, cephalosporins and PBP 2 isoforms were analysed.\(^2\)\(^3\) MICs of β-lactam antibiotics for each strain were previously determined by the agar dilution method.\(^2\) Namely, for ATCC 19424, MICs of penicillin G (Sigma-Aldrich, St Louis, MO, USA), cefdinir (Fujisawa, Osaka, Japan), cefixime (Fujisawa) and ceftriaxone (Roche Diagnostics, Tokyo, Japan) were 0.03, 0.004, 0.002 and <0.001 mg/L. For GU01-29, MICs of penicillin G, cefdinir, cefixime and ceftriaxone were 0.125, 0.015, 0.015 and 0.004 mg/L. Compared with PBP 2 from ATCC 19424, GU01-29 had an Asp-345a insertion. In addition, for GU01-89, MICs of penicillin G, cefdinir, cefixime and ceftriaxone were 4.0, 1.0, 0.5 and 0.25 mg/L. PBP 2 from GU01-89 possessed a mosaic structure composed of fragments of PBP 2 from *N. cinerea* and *N. phflava*.

**Construction of recombinant transfer vectors**

DNAs were extracted from the strains by the phenol–chloroform method.\(^2\)\(^4\) Amplification of the *penA* gene was performed by PCR with primers *penA*-F2 (5′-GAATCTTGATTAAGCGAATA TAAGCCG-3′) (Operon Biotechnologies, Huntsville, AL, USA) and *penA*-R3 (5′-GCAGCGCCTTTAAGACGGCTTGTATGTTGACGCG-3′) (Operon Biotechnologies). PCR products were cloned into the cloning vector pCR2.1 (Invitrogen, San Diego, CA, USA), to generate *penA*-pCR2.1 in TOP10 (Invitrogen), *penA*-pCR2.1 was digested with EcoRI and NotI, and the DNA fragments were ligated to transfer vector pAcHHLT-A (BD Biosciences, San Jose, CA, USA), which was digested with EcoRI and NotI, to produce *penA*-pAcHHLT-A in TOP10. The transfer vector pAcHHLT-A encoded an N-terminal 6×His tag and an extended multiple cloning site.

**Generation of recombinant baculoviruses and expression of PBP 2**

S97 cells (Invitrogen) derived from an insect cell line were co-transfected with linearized baculovirus DNA (BD Biosciences) and *penA*-pAcHHLT-A by the lipofectin-mediated method.\(^2\)\(^5\) The cells were incubated at 26.5°C in SF-900II medium (GIBCO), and recombinant baculoviruses were generated.\(^2\)\(^6\) Each recombinant virus was plaque-purified three times.\(^2\)\(^6\) In addition to the S97 cells, we used an insect cell line from *Trichoplusia ni*, BTL-Tn 5B1-4 (Tn5) (Invitrogen), for large-scale expression. Tn5 cells were infected with recombinant baculoviruses at a multiplicity of infection of 10 and were incubated in Excell 405 medium (JRH Biosciences, Lenexa, KS, USA) for 3 days at 26.5°C until an extensive cytopathic effect was observed.

**Purification of recombinant PBP 2**

Three types of recombinant PBP 2 were purified with a 6×His expression and purification kit (BD Biosciences). Infected 9.0×10⁷ Tn5 cells were harvested and centrifuged at 1000×g for 15 min. The cell pellet was resuspended in ice-cold insect cell lysis buffer (10 mM Tris, pH 7.5, 130 mM NaCl, 2% Triton X-100, 10 mM NaF, 10 mM NaPi, 10 mM NaPPi) containing a protease inhibitor cocktail (16 mg/L benzamidine HCl, 10 mg/L phenanthroline, 10 mg/L aprotinin, 10 mg/L leupeptin, 10 mg/L pepstatin A and 1 mM PMSF). Five millilitres of cell lysis buffer was used, and the lysed cells were maintained on ice for 45 min. This was followed by filtration through a 0.22 μm filter to obtain a clear lysate containing recombinant 6×His PBP 2. The lysate was added at a one-tenth volume to Ni-NTA agarose and incubated at 4°C for 1 h on a rocking platform. The bead slurry was washed four times with 10 bead volumes of 6×His wash buffer (50 mM Na-phosphate, 300 mM NaCl, 30 mM imidazole, 10% glycerol, pH 8.0).
Recombinant 6×His PBP 2 was eluted ~0.9 mg with 6×His elution buffer (50 mM Na-phosphate, 300 mM NaCl, 300 mM imidazole, 10% glycerol, pH 6.0). Eluted proteins were confirmed to be recombinant 6×His PBP 2 by SDS–PAGE and Pro-Q® Sapphire 532 Oligohistidine Gel Stain (Molecular Probes, Eugene, OR, USA).

Fluorescent penicillin binding
Each of the recombinant PBP 2s (2.5 μg) from N. gonorrhoeae (ATCC 19424) and from the two clinical strains of N. gonorrhoeae (GU01-29 and GU01-89) was incubated with BOCILLIN™ FL penicillin (Molecular Probes) in the presence or the absence of a 1000-fold excess of unlabelled penicillin V (Sigma-Aldrich) for 10 min at 30°C. PBP 2s were then solubilized with sarcosyl and resolved by SDS–PAGE; then, the gels were analysed by the luminescent image analyser LAS-3000. PBP 2 competition assays were performed in triplicate. IC₅₀ was defined as the concentration of unlabelled antibiotic that decreased binding of BOCILLIN™ FL penicillin to a PBP 2 by 50%.

Results

Expression and purification of PBP 2
Proteins of 64 kDa molecular weight, consistent with that of PBP 2s, were expressed in Tn5 cells infected with baculovirus with the penA gene amplified from ATCC 19424, GU01-29 and GU01-89. The proteins were purified from insect cell lysates by the 6×His tag. Eluted proteins were confirmed to be recombinant 6×His PBP 2 by SDS–PAGE and Pro-Q® Sapphire 532 Oligohistidine Gel Stain.

Figure 1. Plot of BOCILLIN™ FL penicillin binding to recombinant PBP 2 from ATCC 19424 (a), GU01-29 (b) and GU01-89 (c). Each of the recombinant PBP 2s (2.5 μg) was incubated with BOCILLIN™ FL penicillin in the presence or absence of a 1000-fold excess of unlabelled penicillin V for 10 min at 30°C. PBP 2s were then solubilized with sarcosyl, and labelled PBP 2s were resolved by SDS–PAGE. The gels were analysed by a luminescent image analyser. The insert shows the Scatchard plot analysis. The experiments were performed in duplicate.
Characterization of binding of fluorescent penicillin to PBP 2

BOCILLINTM FL penicillin binding to recombinant PBP 2 at 30°C reached equilibrium after 10 min. Thus, further binding experiments were performed at 30°C for 10 min. Incubation of increasing concentrations of BOCILLINTM FL penicillin with recombinant PBP 2s showed that the specific binding consisted of a saturable component (Figure 1). The levels of non-specific binding were 0% of the total binding. Scatchard plot analysis of the binding data revealed the presence of affinity sites for BOCILLINTM FL penicillin on recombinant PBP 2s (Figure 1). The \( K_d \) values of the affinity sites from ATCC 19424, GU01-29 and GU01-89 were 3.30, 5.94 and 11.57, respectively.

Affinities of recombinant PBP 2s for β-lactam antibiotics

The affinity of each recombinant PBP 2s for penicillin V, cefdinir, cefixime and ceftriaxone was determined by a PBP 2 competition assay, in which BOCILLINTM FL penicillin competed with β-lactam antibiotics bound to PBP 2s. Complexes of the PBP 2 from ATCC 19424, GU01-29 and GU01-89 and BOCILLINTM FL penicillin showed fluorescent bands on SDS–PAGE gels (Figure 2). IC\(_{50}\) values of 76.84, 226.82 and 640.04 pmol for PBPs from ATCC 19424, GU01-29 and GU01-89, respectively, were required for penicillin V (Figure 3). IC\(_{50}\) values of 16.38, 10.81 and 350.51 pmol were also required for cefdinir. IC\(_{50}\) values of 9.91, 13.73 and 29.41 pmol were required for cefixime. IC\(_{50}\) values of 5.22, 7.13 and 7.27 pmol were required for ceftriaxone. The affinity of mosaic-structure recombinant PBP 2 from GU01-89 for cefdinir or cefixime was lower than that of the non-mosaic-structure recombinant PBP 2s from ATCC 19424 and GU01-29. The affinity of the mosaic-structure recombinant PBP 2 for ceftriaxone was not changed, compared with that of the non-mosaic-structure recombinant PBP 2s from ATCC 19424 and GU01-29.

![Figure 2](image-url)

Figure 2. Detection of recombinant PBP 2 from ATCC 19424 (a), GU01-29 (b) and GU01-89 (c) determined by the PBP 2 competition assay. PBP 2s were incubated with BOCILLINTM FL penicillin (100 pmol) and increasing concentrations of penicillin V, cefdinir, cefixime or ceftriaxone (1–10\(^5\) pmol) or with solvent alone (lanes N) for 10 min at 30°C. Complexes of antibiotics and PBP 2s were then solubilized with sarcosyl, and labelled PBP 2s were resolved by SDS–PAGE. The gels were analysed by a luminescent image analyser.

Discussion

In our previous study, we identified mosaic PBP 2 in clinical isolates of \( N. \) gonorrhoeae that was composed of fragments from PBP 2 of \( N. \) cinerea and \( N. \) perflava.\(^{23} \) The interspecies horizontal exchange of the \( penA \) genes or gene parts has been observed among penicillin-resistant strains of \( N. \) gonorrhoeae,\(^{28,29} \) \( N. \) lactamica,\(^{30} \) \( N. \) meningitidis\(^{31} \) and \( N. \) mucosa.\(^{32} \) Commensal species such as \( N. \) flavescens and \( N. \) cinerea, which are intrinsically more resistant to penicillin than pathogenic species, have been identified as the donors in these interspecies exchanges.\(^{29,32} \) In the present study, this mosaic PBP 2 was significantly associated with decreased susceptibility to oral cephalosporins. Alteration in the affinity of PBPs for oral cephalosporins may be associated with the acquisition of resistance to these agents. However, no studies have appeared on the altered affinity of mosaic PBP 2 for cephalosporins. One reason may be that it is difficult to culture large amounts of \( N. \) gonorrhoeae in liquid broth and to obtain large quantities of a membrane fraction, including PBPs. In the present study, we expressed PBP 2 in insect cells with recombinant baculovirus and purified non-mosaic-structure recombinant PBP 2s and mosaic-structure recombinant PBP 2. This approach allowed us to determine the affinity of mosaic-structure recombinant PBP 2 for cephalosporins.

In the present study, the \( K_d \) values showed that the affinity of BOCILLINTM FL penicillin for mosaic-structure recombinant PBP 2 was lower than that of non-mosaic-structure recombinant PBP 2s from ATCC 19424 and GU01-29. Furthermore, mosaic-structure recombinant PBP 2 from GU01-89 exhibited IC\(_{50}\) values 20 times higher for cefdinir and 3 times higher for cefixime than that of non-mosaic-structure recombinant PBP 2 from ATCC 19424. In spite of the decreased affinity of BOCILLINTM FL penicillin for mosaic PBP 2, the affinity of cefdinir and cefixime for this type of PBP 2 was decreased in the PBP 2 competition assay. These results indicated that the extent of the decrease in the affinity of mosaic PBP 2 for cefdinir and cefixime could be greater than that for BOCILLINTM FL penicillin. Therefore, the affinity of mosaic-structure recombinant PBP 2 from GU01-89 for oral cephalosporins was lower than that of non-mosaic-structure recombinant PBP 2s.

In a previous study, we sequenced the \( penA \) gene in 70 clinical isolates of \( N. \) gonorrhoeae and found this type of mosaic PBP 2 in 47 isolates.\(^{23} \) All of the isolates with mosaic PBP 2, including GU01-89, exhibited cefdinir MICs in the range of 0.25–2 mg/L, whereas the remaining 23 isolates, all possessing PBP 2 with Asp-345a or with Asp-345a and additional amino acid alterations of one to six substitutions, showed cefdinir MICs in the range of 0.015–0.125 mg/L. With respect to cefixime, 45 of 47 isolates with mosaic PBP 2 showed MICs in the range of 0.25–1 mg/L, and the other two showed MICs of 0.125 mg/L. The remaining 23 isolates with non-mosaic PBP 2 showed cefixime MICs in the range of 0.015–0.125 mg/L. The clinical isolates were distinctly categorized into oral
cephalosporin-resistant and -susceptible types based on the presence or the absence of mosaic PBP 2.

It should be noted that ceftriaxone was more active against ATCC 19424 with penicillin-susceptible non-mosaic-structure recombinant PBP 2 than cefdinir or cefixime and showed a lower IC50 value for the non-mosaic-structure recombinant PBP 2 than cefdinir or cefixime. The affinity of non-mosaic-structure recombinant PBP 2 with Asp-345a and the affinity of mosaic-structure recombinant PBP 2 for ceftriaxone were not changed, compared with non-mosaic-structure recombinant PBP 2 from ATCC 19424. The results of our previous study showed that the ceftriaxone MICs for isolates with mosaic PBP 2, ranging from 0.015 to 0.25 mg/L, were significantly greater than those for isolates with non-mosaic PBP 2, which ranged from 0.004 to 0.125 mg/L. However, the distribution of the former MICs overlapped that of the latter MICs, and some isolates with mosaic PBP 2 showed lower ceftriaxone MICs than those with non-mosaic PBP 2. The findings of our present study, together with those of our previous study, indicate that the mosaic form decreases the affinity of PBP 2 for cephalosporins, contributing to decreased susceptibility to these antibiotics. However, the same IC50 value of mosaic-structure recombinant PBP 2 for ceftriaxone was observed when compared with that of non-mosaic-structure recombinant PBP 2 with Asp-345a. The isolates with mosaic PBP 2 and non-mosaic PBP 2 exhibited wide ranges of ceftriaxone MICs that overlapped with each other. In clinical isolates of N. gonorrhoeae, the susceptibilities of cephalosporins, particularly ceftriaxone, appeared to be determined not only by alterations in PBP 2 but also by other mechanisms. For most β-lactam antibiotics, PBP 2 is the potential target for killing cells of N. gonorrhoeae. However, several antibiotics exhibit high levels of binding to PBP 1 as well as PBP 2 in both susceptible and resistant strains. Therefore, the findings of the present study also suggest that the acquisition of high levels of resistance to cephalosporins in clinical isolates may be accompanied by an additional change in PBP 1 affinity and/or other resistance mechanisms.

**Figure 3.** Affinity of recombinant PBP 2 from ATCC 19424 (a), GU01-29 (b) and GU01-89 (c) for penicillin V, cefdinir, cefixime and ceftriaxone determined by the PBP 2 competition assay. IC50 values of 76.84, 226.82 and 640.04 pmol for ATCC 19424, GU01-29 and GU01-89, respectively, were required for penicillin V. IC50 values of 16.38, 10.81 and 350.51 pmol were required for cefdinir. IC50 values of 9.91, 13.73 and 29.41 pmol were required for cefixime. IC50 values of 5.22, 7.13 and 7.27 pmol were required for ceftriaxone.
We suggest that the decreased affinity of mosaic-structure recombinant PBP 2 for cephalosporins may contribute to decreased susceptibility to these antibiotics. The present study was limited by the fact that other PBPs were not analysed for affinity for cephalosporins and by the fact that the quantities of PBPs, including mosaic PBP 2, expressed in the cells were not assessed because recombinant PBP 2s were assessed instead of membrane fractions of N. gonorrhoeae. We also did not analyse porin permeability or efflux pump expression in this study. Other mechanisms may be involved in the decreased susceptibility to these agents. Nevertheless, this is the first study to analyse the affinity of mosaic-structure recombinant PBP 2 to cephalosporins. The results should prove useful for elucidating the mechanisms of cephalosporin resistance in clinical strains of N. gonorrhoeae.

Acknowledgements

We thank Tian-Cheng Li, Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan, for guiding us in the use of the baculovirus expression vector system, as well as our colleagues Takamaro Miyazawa and Chikako Sakata at the Research and Development Department, Mitsubishi Kagaku Bio-Clinical Laboratories, Inc., Tokyo, Japan, for their technical advice and assistance.

Transparency declarations

None to declare.

References


