Inhibitory action of telithromycin against Shiga toxin and endotoxin

Saori Nakagawa,1 Seiichi Kojio,1 Ikue Taneike, Nobuhiro Iwakura, Yukiko Tamura, Koji Kushiya, Fumio Gondaira, and Tatsuo Yamamoto*

Division of Bacteriology, Department of Infectious Disease Control and International Medicine, Niigata University Graduate School of Medical and Dental Sciences, 757 Ichibanchou, Asahimachidori, Niigata, Japan

Received 20 September 2003

Abstract

Shiga toxin (Stx)-producing Escherichia coli (STEC) is associated with hemolytic uremic syndrome (HUS). High inflammatory cytokine [interleukin (IL)-6 and IL-8] levels and low anti-inflammatory cytokine (IL-10) levels are indicators of a high risk for developing HUS in STEC-infected children. In this study, we investigated inhibitory action of telithromycin, a ketolide, against STEC and against Stx and lipopolysaccharide (LPS). Telithromycin inhibited in vitro STEC growth without inducing Stx phage, in marked contrast to norfloxacin. Stx markedly induced inflammatory (but not anti-inflammatory) cytokine production in human peripheral blood monocytes, while LPS induced both inflammatory and anti-inflammatory cytokine production. Telithromycin selectively inhibited the IL-6 and IL-8 production from Stx-stimulated (but not LPS-stimulated) monocytes. The drug did not significantly inhibit IL-10 production. Our data suggest that Stx plays a crucial role in the stimulation of inflammatory cytokines and such inflammatory response is inhibited by telithromycin, an anti-bacterial agent.

Keywords: Telithromycin; Escherichia coli; Phage; Shiga toxin; Endotoxin; Cytokine

Telithromycin (HMR3647) belongs to the ketolide group, which represents a new class of macrolide-like antibiotics [1]. Telithromycin possesses a ketone, instead of a cladinose sugar, at position 3 of a 14-member macrolactone ring and a carbamate side chain at positions 11–12. Due to its unique structural characteristics, telithromycin exhibits excellent activity on macrolide-resistant Streptococcus pneumoniae in vitro [2].

Macrolides, including erythromycin and clarithromycin (possessing a 14-member macrolactone ring) or azithromycin (possessing a 15-member macrolactone ring), demonstrate anti-inflammatory properties; e.g., they reduce the release of inflammatory cytokines including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and IL-8 in, e.g., lipopolysaccharide (LPS)-stimulated human monocytes [3].

Shiga toxin (Stx)-producing Escherichia coli (STEC) continues to be an important food-borne bacterial pathogen threatening public health [4,5]. Its infection is closely associated with serious systemic complications such as hemolytic uremic syndrome (HUS) and encephalopathy, especially in the young and elderly [6,7]. The host’s inflammatory responses, such as increased levels of TNF-α, IL-1β, IL-6, and IL-8, are involved in the development of HUS [8–10]. Stx, similar to LPS, induces the production of inflammatory cytokines in human monocytes.

Stx is encoded by lysogenic phage(s), and its production is stimulated by some antimicrobial agents, such as a derivative of fluoroquinolones (e.g., norfloxacin) [11–13]. In this study, we investigated the inhibitory effect of telithromycin on STEC growth and Stx phage induction, and also on the release of cytokines from Stx- and LPS-stimulated human peripheral blood monocytes.

Materials and methods

STEC strains. The STEC strains (51 strains) used in this study included 18 strains of serotype O157:H7 derived from 9 outbreaks in Japan in 1996 [14], 19 strains of serotype O157:H7 derived from

* Corresponding author. Fax: +81-25-227-0762. E-mail address: tatsuoy@med.niigata-u.ac.jp (T. Yamamoto).
1 The authors S.N. and S.K. contributed equally to this work.

Antimicrobial agents. Telithromycin (Aventis Pharma, Tokyo), azithromycin (Pfizer Pharmaceuticals, Tokyo), clarithromycin (Taiho Pharmaceutical, Tokyo), roxithromycin (Aventis Pharma), erythromycin (Shionogi, Osaka), and norfloxacin (Daichi Pharmaceutical, Tokyo) were gifts from their manufacturers.

Susceptibility testing. Susceptibility testing of bacterial strains was done with the agar dilution method with Mueller–Hinton agar according to standard procedures [16].

Stx-converting phage induction. Phage induction was performed essentially as described previously [11,12]. STEC strain 1076 was grown to log phase (ca. 5.0 \times 10^8 CFU/ml) in LB broth (Difco Laboratories, Detroit, MI) containing 10 mM CaCl_2 at 37°C and incubated for 30 min in the presence or absence of various concentrations of antimicrobial agents. The bacterial cells were washed and resuspended in the same volume of fresh LB broth, followed by incubation for 2 h. The resultant bacterial culture was divided into two parts. One part was centrifuged and the supernatant was subjected to phage titration using E. coli strain C600 as an indicator strain. The phage plaques were examined for the Stx gene by PCR assay [17] to confirm that they were all from Stx phage. The remaining part of the bacterial culture was subjected to the reverse passive latex agglutination test using a VT detection kit (Denka Seiken, Tokyo). The Stx titers (the levels of Stx production) represented the highest dilution to yield positive results.

Preparation of Stx. Stx1 produced from E. coli strain 87-27 (carrying the cloned Stx1 gene) and Stx2 produced from E. coli strain Tp8 (carrying the cloned Stx2 gene) were purified to homogeneity as described previously [18]. Purified Stx1 and Stx2 (0.2 mg protein per ml) were stored at 4°C in phosphate-buffered saline (PBS). The Stx1 and Stx2 preparations contained no detectable endotoxin (less than 0.05 endotoxin units per ml) contamination as determined by a limulus amebocyte lysate assay.

Preparation of human peripheral blood monocytes. Peripheral blood from eight healthy adults was used in this study. Human peripheral blood mononuclear cells were prepared by using ficoll-conray (Amersham Biosciences, NJ) gradient centrifugation, as previously described [19]. Mononuclear cells were suspended in RPMI-1640 culture medium (Gibco-BRL, Grand Islands, NY) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL) at 1 \times 10^7 cells/ml. For preparation of monocytes, plastic-nonadherent cells were removed after 2 h of incubation at 37°C and then adherent monocytes were collected [19].

Cytokine induction by Stx- or LPS-stimulated monocytes. Monocytes were cultured in 24-well microtiter plates at a density of 5 \times 10^5 cells/ml in RPMI-1640 supplemented with 10% FBS at 37°C. Stx (at a concentration of 5 mg/ml) or LPS (E. coli O55:B5, Sigma, St. Louis, MO) (at a concentration of 20 mg/ml) was added to wells containing monocytes and incubated for 18 h, in the presence or absence of drugs (at a concentration of 2.5, 5 or 10 mg/ml). Levels of immunoreactive TNF-α, IL-1β, IL-6, IL-8, and IL-10 in culture supernatants were measured by enzyme-linked immunosorbent assays (ELISA) (Genzyme Technica, Minneapolis, MN).

Statistical analysis. Data were evaluated by Mann–Whitney U test. The level of significance was a p value less than 0.05.

Results

In vitro activity against STEC

The minimum inhibitory concentrations (MICs) of the antimicrobial agents against the STEC strains are summarized in Table 1. Among the macrolides and ketolide tested, azithromycin showed the greatest activity (MIC90, 8 µg/ml). This was followed by telithromycin (MIC90, 16 µg/ml), clarithromycin (MIC90, 32 µg/ml), erythromycin (MIC90, 64 µg/ml), and roxithromycin (MIC90, ≥256 µg/ml). The activity of norfloxacin (a newer fluoroquinolone) was greater than that of azithromycin or telithromycin (MIC90, 0.13 µg/ml).

Effect on Stx phage induction

Norfloxacin markedly induced Stx2 phage from Stx2+ STEC strain 1076 at a concentration of 0.1 µg/ml or more (Fig. 1A), which was close to the MIC value of the test strain (0.06 µg/ml). The number of plaque forming units was 10^4–10^5-fold greater, as compared with the level of spontaneous phage induction. The frequency of phage induction in the presence of telithromycin was similar to or even less than that of spontaneous phage induction (Fig. 1A), suggesting that telithromycin did not induce Stx phage.

<table>
<thead>
<tr>
<th>antimicrobial agents</th>
<th>MICa (µg/ml)</th>
<th>50%</th>
<th>90%</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azithromycin</td>
<td>4</td>
<td>8</td>
<td>2–8</td>
<td></td>
</tr>
<tr>
<td>Telithromycin</td>
<td>8</td>
<td>16</td>
<td>2–16</td>
<td></td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>32</td>
<td>32</td>
<td>16–64</td>
<td></td>
</tr>
<tr>
<td>Erythromycin</td>
<td>32</td>
<td>64</td>
<td>32–64</td>
<td></td>
</tr>
<tr>
<td>Roxithromycin</td>
<td>128</td>
<td>≥256</td>
<td>64–256</td>
<td></td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>0.13</td>
<td>0.13</td>
<td>0.06–0.5</td>
<td></td>
</tr>
</tbody>
</table>

*50% and 90% MICs required to inhibit the growth of 50% and 90% of the strains tested, respectively. Range of MICs for the test strains are also shown.

Fig. 1. Effects of antimicrobial agents on Stx phage induction and Stx production in STEC. STEC strain 1076 (producing Stx2) was treated with the indicated concentrations of antimicrobial agents for 30 min, followed by 2 h incubation in drug-free medium. In (A), the number of Stx2 phages induced was determined. Antimicrobial agents used were norfloxacin (open square) and telithromycin (closed square). PFU, the numbers of plaque phages that developed in the LB soft agar plates. In (B), Stx2 levels in the bacterial culture were examined. Antimicrobial agents used were as follows: open bars, norfloxacin; shaded bars, telithromycin.
In accordance with phage induction, norfloxacin induced marked Stx production at a concentration of 0.1 g/ml (Fig. 1B). The Stx titer increased by 512-fold compared with that observed in the absence of antimicrobial agents. In marked contrast, telithromycin did not induce Stx production (Fig. 1B).

Diverse cytokine production in Stx- and LPS-stimulated human peripheral blood monocytes

Human monocytes were stimulated with Stx or LPS for 18 h. Both Stx1 and Stx2 stimulated the production of TNF-α, IL-1β, IL-6, and IL-8 from human monocytes (Fig. 2). In the case of IL-10, however, the production levels were extremely low (Fig. 2). In marked contrast, LPS stimulated the production of TNF-α, IL-1β, IL-6, IL-8 as well as IL-10 from human monocytes (Fig. 2).

Inhibition of the cytokine production by Stx-stimulated human monocytes

Telithromycin was examined for its effects on the production of cytokines (TNF-α, IL-1β, IL-6, and IL-8) from Stx2-stimulated human monocytes (Fig. 3). Addition of telithromycin to the monocyte reaction mixtures resulted in significant suppression of cytokine production ($p < 0.05$) (Fig. 3). Very similar results as shown in...

![Graph](image-url)
Fig. 3 were obtained even when Stx1 was used instead of Stx2 (data not shown).

The three macrolides, erythromycin, clarithromycin, and azithromycin, were used as reference immunomodulators. The inhibitory effect of telithromycin was much greater than that of erythromycin and was similar to or even greater (especially in IL-6) than that of azithromycin (Fig. 3).

Inhibition of the cytokine production by LPS-stimulated human monocytes

When telithromycin was examined for its effects on the production of cytokines (TNF-α, IL-1β, IL-6, IL-8, and IL-10) from LPS-stimulated human monocytes, significant inhibition was observed only with TNF-α at 2.5 μg/ml or higher (p < 0.05), as shown in Fig. 4. This inhibitory effect of telithromycin on TNF-α production was greater than that of erythromycin (p < 0.05). No significant inhibition was observed with IL-1β, IL-6, IL-8 or IL-10.

Discussion

We demonstrated in this study that telithromycin has relatively strong MIC values for STEC strains, compared with those of macrolides tested. Norfloxacin (a derivative of fluoroquinolones) had a superior activity, but this drug did induce Stx phage from STEC and caused a higher level of Stx production. In sharp contrast, telithromycin did not induce Stx phage.

Stx prophages are induced with agents such as UV or fluoroquinolones that damage DNA or inhibit DNA replication [12,13,20]. This is the case even in vivo; e.g., administration of ciprofloxacin (a derivative of fluoroquinolones) caused Stx phage induction as well as Stx production from STEC and death in mice [13]. In contrast, the primary target of action for telithromycin is protein synthesis [21]. The different mechanisms of action of norfloxacin vs. telithromycin most likely explain differences in phage induction.

In this study, we also examined the inhibitory effect of telithromycin on the release of cytokines from bacterial toxin-stimulated human peripheral blood monocytes. It was found that LPS stimulated the production of both inflammatory cytokines (TNF-α, IL-1β, IL-6, and IL-8) and an anti-inflammatory cytokine (IL-10) from human monocytes, while Stx stimulated inflammatory cytokines (TNF-α, IL-1β, IL-6, and IL-8) rather than IL-10. High ratios of serum inflammatory cytokine levels (IL-6/IL-10 or IL-8/IL-10) are indicators of a risk for developing HUS in STEC-infected children [10,22]. Therefore, the unique cytokine pattern observed with Stx (high levels of IL-6 and IL-8 and relatively low levels of IL-10) well reflects the serum cytokine patterns in HUS patients, indicating that Stx plays a crucial role in the stimulation of inflammatory cytokines.

This study clearly demonstrated that telithromycin inhibits the production of inflammatory cytokines (TNF-α, IL-1β, IL-6, and IL-8) from Stx-stimulated human peripheral blood monocytes. This inhibitory effect was obvious at a concentration as low as 2.5 μg/ml and was much greater than that of erythromycin and
similar to or even greater (especially in IL-6 production) than that of azithromycin.

Since IL-6 plays a role in platelet aggregation in HUS development [23], the inhibitory effect of telithromycin in Stx-mediated IL-6 production, which is superior to the effect of azithromycin, is noted as a unique characteristic of the drug. This observation may be consistent with the previous data that in *S. pneumoniae* experimental pneumonia in mice, ketolide administration drastically decreased IL-6 levels in blood and lung tissue [24]; in this mouse model, the drug also drastically increased survival rates of infected mice.

The difference in the inhibitory activities, regarding the cytokine production, among four macrolide and ketolide antimicrobial agents examined (including telithromycin) could be explained by the diverse levels of uptake and cellular accumulation of the agents. Ketolide antimicrobial agents (including telithromycin) more strongly accumulate within cells than macrolide antimicrobial agents such as roxithromycin [25–27]. Azithromycin also accumulates strongly within cells compared with erythromycin [28]. Strong ketolide (or macrolide) accumulation within cells may cause the reduction of the cytokine production.

In the case of LPS-stimulated human peripheral blood monocytes, however, telithromycin exhibited significant inhibition only for TNF-α, but not for IL-1β, IL-6, and IL-8. It is noteworthy that telithromycin did not significantly inhibit anti-inflammatory cytokine (IL-10) production.

We previously reported that anisodamine (an alkaloid extracted from a Chinese herb) inhibits TNF-α, IL-1β, and IL-8 production by Stx1-stimulated human peripheral blood monocytes, and increases the survival of Stx1-treated mice [19]. Anisodamine, however, had no effects on the in vitro growth of STEC strains (unpublished data).

In contrast, telithromycin showed an activity against bacterial growth of STEC belonging to various serotypes in vitro. Telithromycin could interfere with STEC infections in two stages: (i) the initial bacterial colonization in the intestines by inhibiting bacterial growth (without inducing Stx phages) and (ii) the inflammatory cytokine responses by inhibiting cytokine production from Stx-stimulated monocytes (and also TNF-α production from LPS-stimulated monocytes).

The MIC values of telithromycin for STEC are relatively high. However, erythromycin with relatively high MIC values (MIC₉₀, 4 μg/ml) for *Campylobacter jejuni* has been recommended for treatment of *Campylobacter enterocolitis* [29,30]. Telithromycin may also exhibit anti-STEC activity.

STEC infection continues to threaten public health. In Japan, large outbreaks occurred in 1996 with 17,877 people infected and 12 died (of fatalities, 7 were children). And in 2002, large infections occurred with nine fatalities. Ketolide (such as telithromycin) and macrolides (such as azithromycin) may be a useful anti-STEC agent, acting against both bacterial growth (without inducing Stx phage) and subsequent toxin-induced, HUS-related inflammatory response. The merit of telithromycin demonstrated in this study is its high ability to reduce the Stx-stimulated IL-6 production, compared with macrolides.

Acknowledgments

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, and a grant (97-1) from the Organization for Pharmaceutical Safety and Research (OPSR), Japan.

References


