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ORIGINAL ARTICLE

Antimicrobial efficacies of several antibiotics against uterine cervicitis caused by *Mycoplasma genitalium*

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Abstract *Mycoplasma genitalium* has been shown to be one of the pathogens responsible for uterine cervicitis by many studies. However, there are no clinical recommendations for treating *M. genitalium*-positive uterine cervicitis. Our study retrospectively investigated the antimicrobial efficacies of several antibiotics against uterine cervicitis caused by *M. genitalium*. We studied a total of 257 women with *M. genitalium*-positive uterine cervicitis, except for those with chlamydial and gonococcal infections, who were treated with one of the following antibacterial therapies: azithromycin extended release formulation (AZM-SR) 2 g single dose, azithromycin (AZM) 1 g single dose, clarithromycin (CAM) 400 mg/day for 7 days, CAM 400 mg/day for 14 days, moxifloxacin (MFLX) 400 mg/day for 7 days, MFLX 400 mg/day for 14 days, levofloxacin (LVFX) 500 mg/day for 7 days, LVFX 500 mg/day for 14 days, sitafloxacin (STFX) 200 mg/day for 7 days, and STFX 200 mg/day for 14 days. A PCR-based assay was performed to evaluate the microbiological efficacy of eradication in these patients. *M. genitalium* was eradicated from the uterine cervix in 19 of the 21 (90.5%) patients treated with AZM-SR 2 g single dose, in 38 of the 42 (90.5%) patients treated with MFLX 400 mg/day for 7 days, in 42 of the 42 (100%) patients treated with MFLX 400 mg/day for 14 days, and in 12 of the 13 (92.3%) patients treated with STFX 200 mg/day for 14 days. In conclusion, AZM-SR 2 g single dose, MFLX 400 mg/day for 14 days, and STFX 200 mg/day for 14 days would each be an effective treatment for *M. genitalium* infection.

Keywords *Mycoplasma genitalium* · Uterine cervicitis · Azithromycin · Moxifloxacin · Sitafloxacin

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Introduction

Mycoplasma genitalium was initially isolated from two patients with nongonococcal urethritis by Taylor-Robinson et al. in 1980 [1]. Its inoculation intraurethrally into male chimpanzees caused urethritis with an antibody response. These findings suggested that this mycoplasma could be a causative organism of nongonococcal urethritis in humans [2]. However, it has been so difficult to culture *M. genitalium* that its pathogenic role in urethritis has not been well established [3–9].

With recent advances in molecular technology, particularly polymerase chain reaction, it is now possible to detect *M. genitalium* in clinical specimens. *M. genitalium* is also a cause of female genital infection in women, and has been detected in women [2, 10–12]. In other studies, neither clinical signs nor patient symptoms were associated with *M. genitalium* infection [13, 14]. In one study, the prevalence of *M. genitalium* infection was 26.3% by PCR assay [12]. In another study, *M. genitalium* was detected in 22.4% of subjects by PCR assay [15]. We have reported that a significantly greater prevalence of *M. genitalium* was demonstrated in Japanese women with female genital infection, which would suggest that *M. genitalium* might play a pathogenic or infection-promotor role in female genital infection [16].

Many studies have shown *M. genitalium* to be one of the pathogens responsible for uterine cervicitis [2, 10–13, 15]. However, there are no clinical recommendations for treating *M. genitalium*-positive uterine cervicitis [17]. The study described in the present work investigated the clinical efficacies of various antibiotics against uterine cervicitis caused by *M. genitalium*.

Patients and methods

Study design

This was a retrospective, single-center, study focusing on the period from January 2008 to August 2010. Women patients with *Mycoplasma genitalium*-positive uterine cervicitis received the following antibacterial therapies: azithromycin extended release formulation (AZM-SR) 2 g single dose, azithromycin (AZM) 1 g single dose, clarithromycin (CAM) 400 mg/day for 7 days, CAM 400 mg/day for 14 days, moxifloxacin (MFLX) 400 mg/day for 7 days, MFLX 400 mg/day for 14 days, levofloxacin (LVFX) 500 mg/day for 7 days, LVFX 500 mg/day for 14 days, sitafloxacin (STFX) 200 mg/day for 7 days, or STFX 200 mg/day for 14 days.

Each patient had two visits: a baseline visit (day 1) and an EOS (end of study: 14 days after end of treatment) evaluation visit. PCR-based assay was performed to evaluate the microbiological efficacy of eradication in these patients at the baseline and EOS visits.

The protocol was approved by an institutional review board at Izumi Ladies Clinic, and the study was conducted in compliance with the ethical principles of the Declaration of Helsinki. All patients provided written informed consent before initiating the study procedure.

Patients

The study included women aged 18–42 years with uterine cervicitis diagnosed as *M. genitalium*-positive by PCR-based assay. Patients had discharge from the cervix with clinical symptoms of cervicitis. Two major diagnostic signs characterize cervicitis: (1) a purulent or mucopurulent endocervical exudate visible in the endocervical canal or on an endocervical swab specimen (commonly referred to as "mucopurulent cervicitis" or cervicitis), and (2) sustained endocervical bleeding easily induced by gentle passage of a cotton swab through the cervical os. Either or both signs could be present. Patients had their *M. genitalium* infection confirmed by PCR-based assay, except in those cases with chlamydial and gonococcal infections. We studied a total of 257 women with *M. genitalium*-positive uterine cervicitis.

Sampling procedure

Two cervical swab specimens were obtained from all patients. After the endocervical canal had been cleaned by removing discharge, cotton swabs were inserted and rubbed against the endocervical canal. The first swab was placed into the PCR transport medium to detect *Neisseria gonorrhoeae* and *Chlamydia trachomatis*. A second swab was placed into 0.5 mL of 10 mmol/L Tris–HCl buffer, pH 8.0, including 1 mmol/L EDTA to perform a PCR-based assay to detect *M. genitalium*.

PCR amplification

DNA preparation and PCR amplification using a semi-nested strategy and Southern blot hybridization analysis was performed as previously described. Specimens for which the 300 bp DNA fragment hybridized to the internal probe were regarded as positive for *M. genitalium*. Gonorrhea was excluded by microscopy or a PCR assay. Prevalence of *C. trachomatis* also was determined by testing the cervical swab specimens using a PCR assay.

Detection of *M. genitalium*

To detect *M. genitalium*, a modified version of the published [18] procedure was used. The nucleotide sequences of the *M. genitalium* were made from oligonucleoside of the 140 kDa adhesion protein gene. MgPa-1 was complementary to the coding strand and its sequence was 5′AGT TGATGAAACCTTAACCCCTTGG3′. MgPa-3 was complementary to the noncoding strand and its sequence was 5′CCGTTGAGGGGTTTTCCATTTTTGC3′. These genes were different from those previously reported in the literature [4, 10, 19–22]. Samples were denatured at 95°C for 60 s, and primers were annealed at 52°C for 50 s and extended at 72°C for 50 s. A total of 35 cycles were performed.

Detection of *N. gonorrhoeae* and *C. trachomatis*

Baseline cervical samples were assessed for *N. gonorrhoeae* and *C. trachomatis* by the PCR method (Amplicor® STD-1, Roche Diagnostics K.K., Japan).

Efficacy endpoint

To assess efficacy, the eradication rate was analyzed by PCR-based assay at the EOS evaluation visit in the overall population treated.

Results

Efficacy endpoint

The eradication rates at the EOS visit were 90.5% (19/21) for AZM-SR 2 g single dose, 85.7% (36/42) for AZM 1 g single dose, 65.0% (13/20) for CAM 400 mg/day for 7 days, 85.0% (17/20) for CAM 400 mg/day for 14 days, 90.5% (38/42) for MFLX 400 mg/day for 7 days, 100% (42/42) for MFLX 400 mg/day for 14 days, 54.5% (12/22) for LVFX 500 mg/day for 7 days, 71.4% (15/21) for LVFX 500 mg/day for 14 days, 78.6% (11/14) for STFX 200 mg/day for 7 days, and 92.3% (12/13) for STFX 200 mg/day for 14 days (Table 1).

Safety

All adverse events experienced by the patients during these antibacterial regimens were digestion-related events. The number of AEs increased with the length of the treatment period (Table 2).

Discussion

We examined the antimicrobial efficacies of various antibiotics for *M. genitalium*-positive uterine cervicitis based on the eradication rate at the EOS visit. The eradication assessments were made according to the results of a PCR-based assay.

In most cases, *M. genitalium* infections are asymptomatic [2, 23, 24]. When the diagnosis of *M. genitalium* infection or the detection of *M. genitalium* is delayed, the disease progresses to salpingitis [25] or pelvic inflammatory disease (PID) [26]. The treatment regimens for *M. genitalium* are not mentioned in the literature, even in the guidelines [17]; descriptions of the recommended treatment used for *Mycoplasma* spp. in actual clinical practice are, however. Falk et al. reported that treating *M. genitalium* with tetracyclines (doxycycline and lymecycline) could not be recommended because of low eradication rates. This report indicated that AZM (5-day course: 500 mg the first day and 250 mg for the following 4 days) could be more efficient than tetracyclines [27].

In our study, the eradication rate of *M. genitalium* by AZM-SR 2 g single dose was 90.5% (19/21) and that achieved by AZM 1 g single dose was 85.7% (36/42). Other studies have reported that the eradication rate of *M. genitalium* by AZM 1 g single dose was 84–85% [28–30]. Almost all patients enrolled in these studies were male patients with urethritis. In our study, AZM 1 g single dose yielded the same level of efficacy as these reported data.

A lot of new-generation fluoroquinolones are now available in Japan. Although some fluoroquinolones showed antibacterial activities against *M. genitalium* in

Table 1 Microbiological efficacy (eradication rates)

Regimen	AZM-SR 2 g	AZM 1 g	CAM 400 mg 7 days	CAM 400 mg 14 days	MFLX 400 mg 7 days	MFLX 400 mg 14 days	LVFX 500 mg 7 days	LVFX 500 mg 14 days	STFX 200 mg 7 days	STFX 200 mg 14 days
Number of patients	21	42	20	20	42	42	22	21	14	13
Number of successful microbiological outcomes/total (%)	19/21 (90.5)	36/42 (85.7)	13/20 (65.0)	17/20 (85.0)	38/42 (90.5)	42/42 (100)	12/22 (54.5)	15/21 (71.4)	11/14 (78.6)	12/13 (92.3)

AZM-SR azithromycin extended release formulation, *AZM* azithromycin, *CAM* clarithromycin, *MFLX* moxifloxacin, *LVFX* levofloxacin, *STFX* sitafloxacin

Table 2 Adverse events

Regimen	AZM-SR 2 g	AZM 1 g	CAM 400 mg 7 days	CAM 400 mg 14 days	MFLX 400 mg 7 days	MFLX 400 mg 14 days	LVFX 500 mg 7 days	LVFX 500 mg 14 days	STFX 200 mg 7 days	STFX 200 mg 14 days
Number of patients	21	42	20	20	42	42	22	21	14	13
Number of AEs (%)										
Diarrhea	8 (38.1)	0	0	1 (5.0)	3 (7.1)	4 (9.5)	0	0	1 (7.1)	4 (30.8)
Loose stool	2 (9.5)	1 (2.4)	0	1 (5.0)	3 (7.1)	5 (11.9)	0	2 (14.3)	2 (14.3)	3 (23.1)

AZM-SR azithromycin extended release formulation, *AZM* azithromycin, *CAM* clarithromycin, *MFLX* moxifloxacin, *LVFX* levofloxacin, *STFX* sitafloxacin

Table 3 MICs for isolated pathogens from Japan [32]

Antibiotic	MIC range (mg/L)	MIC 50%	MIC 90%
Sitafloxacin	0.008–0.125	0.063	0.125
Moxifloxacin	0.016–0.25	0.063	0.125
Gatifloxacin	0.031–0.5	0.25	0.25
Levofloxacin	0.125–2	1	2
Ciprofloxacin	0.063–8	4	8
Norfloxacin	1–64	32	64
Minocycline	0.031–0.25	0.125	0.25
Doxycycline	0.063–1	0.125	0.25
Tetracycline	0.063–2	0.125	0.5
Azithromycin	0.0002–250	0.001	0.002
Clarithromycin	0.0005–128	0.004	0.008

MIC minimum inhibitory concentration

vivo (Table 3), the results for the eradication rates achieved with these were lower than expected.

Currently, assays of *N. gonorrhoeae* and *C. trachomatis* are performed using PCR methods that are optimized so that they can be easily conducted by clinics. Mikamo et al. [31] reported that the appropriate method used and time needed to assess the therapeutic efficacy for *C. trachomatis* infectious STD are PCR and three weeks after treatment, respectively. On the other hand, the PCR-based assay of *M. genitalium* is not common nor commercialized, so it needs more time to detect *M. genitalium* than the commercialized assays of *N. gonorrhoeae* and *C. trachomatis*. As *M. genitalium* causes STD or PID, a commercialized assay is needed for early treatment.

The regimen of AZM 1 g is effective for urethritis and cervicitis caused by *C. trachomatis*, and recommended in the guidelines [16]. Single-dose AZM treatment options (AZM-SR 2 g single dose or AZM 1 g single dose) are favorable for the initial treatment for STD if *C. trachomatis* is detected, whatever the result obtained from the *M. genitalium* PCR. If the EOS assessment after AZM treatment finds any remaining *M. genitalium* by PCR or any clinical symptoms, an alternative treatment such as fluoroquinolones is considered. In particular, if female patients may be pregnant, tetracyclines and fluoroquinolones are alternative options or options after confirming that the patient is not pregnant.

There were some clear limitations to our study. Our study is a retrospective study, and the number of patients included meant that statistically significant results were not obtained. A prospective, large-scale clinical study is needed in order to produced such statistically significant results. *M. genitalium* was only detected in the patients by PCR; no other methods were used, such as culture-based confirmation. Our PCR method for *M. genitalium* was not standardized to each site, and a more appropriate method with high sensitivity and high specificity is required.

In our study, *M. genitalium* was eradicated from the uterine cervix in 19 of the 21 (90.5%) patients treated with AZM-SR 2 g single dose, in 38 of the 42 (90.5%) patients treated with MFLX 400 mg/day for 7 days, in 42 of the 42 (100%) patients treated with MFLX 400 mg/day for 14 days, and in 12 of the 13 (92.3%) patients treated with STFX 200 mg/day for 14 days.

In conclusion, AZM-SR 2 g single dose, MFLX 400 mg/day for 14 days, or STFX 200 mg/day for 14 days are each effective treatments for *M. genitalium* infection.

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Adjuvant effect of Japanese herbal medicines on the mucosal type 1 immune responses to human papillomavirus (HPV) E7 in mice immunized orally with *Lactobacillus*-based therapeutic HPV vaccine in a synergistic manner

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ABSTRACT

The Japanese herbal medicines, Juzen-taiho-to (JTT) and Hochu-ekki-to (HET), have been shown to enhance humoral immune responses to vaccine antigen when used as adjuvants for prophylactic vaccines. However, their adjuvant effect on mucosal cellular immune responses remains unstudied. The precursor lesion of cervical cancer, high-grade CIN that expresses HPV E7 oncoprotein ubiquitously is a target for HPV therapeutic vaccines that elicit mucosal E7-specific type 1 T cell responses. We have demonstrated that oral immunization with recombinant *Lactobacillus casei* expressing HPV16 E7 (LacE7) is more effective in eliciting mucosal E7-specific IFNγ-producing cells than subcutaneous or intramuscular antigen delivery. Here we report the synergistic effect of an oral *Lactobacillus*-based vaccine and Japanese herbal medicines on mucosal immune responses. Oral immunization of mice with LacE7 plus either a Japanese herbal medicine (JTT or HET) or a mucosal adjuvant, heated-labile enterotoxin T subunit (LTB), promotes systemic E7-specific type 1 T cell responses but not mucosal responses. Administration of LacE7 plus either Japanese herbal medicine and LTB enhanced mucosal E7-specific type 1 T cell response to levels approximately 3-fold higher than those after administration of LacE7 alone. Furthermore, secretion of IFNγ and IL-2 into the intestinal lumen was observed after oral administration of LacE7 and was enhanced considerably by the addition of Japanese herbal medicines and LTB. Our data indicated that Japanese herbal medicines, in synergy with *Lactobacillus* and LTB, enhance the mucosal type 1 immune responses to orally immunized antigen. Japanese herbal medicines may be excellent adjuvants for oral *Lactobacillus*-based vaccines and oral immunization of LacE7, HET and LTB may have the potential to elicit extremely high E7-specific mucosal cytotoxic immune response to HPV-associated neoplastic lesions.

1. Introduction

Human papillomavirus (HPV) infection is a major risk factor for the development of cervical cancer which is the second most common cancer among women [1]. HPV prophylactic vaccines hold promise to reduce the worldwide incidence of cervical cancer. However, limitations in current HPV vaccine strategies make the development of HPV therapeutic vaccines for the treatment of HPV-associated lesions essential. HPV E7 is an attractive target protein for HPV therapeutic vaccine strategies that are directed against a precursor lesion of cervical cancer, high-grade cervical intraepithelial neoplasia (CIN) [2]. Many therapeutic vaccines against HPV E7 have been developed and several clinical vaccination trials against high-grade CIN have been completed [3–11]. However, no therapeutic HPV vaccines are yet available. The current vaccine candidates have been shown to elicit systemic cellular immunity after intramuscular or subcutaneous injection and clinical trials have shown cellular immune responses to the vaccines in peripheral monocytes but fail to show local immunity in the cervical mucosa after vaccination. Cervical mucosal lesions may be poorly responsive to systemic cellular immunity since precursor lesions develop in the mucosal epithelium; mucosal intraepithelial lymphocytes (IELs) should be the central effector cells for the elimination of CIN. Lymphocytes involved in the mucosal immune system are found in the inductive sites of organized mucosa-associated lymphoid tissues and in a variety of effector sites such as the mucosa of the intestine, respiratory tract and genital tract [12]. The efficient homing of lymphocytes to the gut is dependent on the homing receptors integrin $\alpha4\beta7$ [13]. Several studies have demonstrated that gut-derived integrin $\alpha4\beta7^+$ lymphocytes subsequently home to the genital mucosa [14–17].

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We have reported previously that the oral *Lactobacillus*-based vaccine expressing HPV16 E7 (LacE7) has substantial potential to be a novel HPV therapeutic vaccine [18]. Oral immunization with LacE7 elicited E7-specific IFNγ-producing cells (T cells with E7-type1 immune responses) among integrin $\alpha4\beta7^+$ mucosal lymphocytes collected from gut mucosa. In our previous study, oral immunization with LacE7 preferentially elicited E7-specific type1 T cell responses in mucosal lymphocytes when compared to splenocytes. Taken together with the data that gut-derived integrin $\alpha4\beta7^+$ T cells home to the cervical mucosa [19], we predicted that vaccine-induced mucosal $CD4^+$ and $CD8^+$ T cells will have antitumor effects on mucosal HPV E7-related neoplastic lesions.

Traditional Chinese herbal medicines and their Japanese counterparts, Japanese herbal medicines, are used not merely to improve weak constitutions but also to suppress many constitutional symptoms. The Japanese herbal medicines, Juzen-taiho-to (JTT) and Hochu-ekki-to (HET), have been reported to exert beneficial effects on various aspects of the immune response [20] and are thought to have great potential as adjuvants for prophylactic vaccination against a variety of microbes [21–23]. JTT's immunomodulatory actions include an enhancement of the mitogenic activity of spleen cells, a promotion of phagocytosis and anti-tumor effect [24,25]. HET activates natural killer cells and macrophages [26,27]. Orally administered HET increases antibody titers against influenza virus in mice immunized with influenza vaccines and promotes secretory IgA production after oral OVA vaccination [28,29].

Viewing the actions of JTT and HET on innate immunity within the intestinal mucosa after oral vaccination, we hypothesized that concurrent oral administration of JTT or HET and LacE7 would enhance mucosal cellular immune responses against HPV16 E7. To address the immunomodulatory effects of JTT or HET on anti-E7 immune responses, mice were given oral JTT or HET in addition to a LacE7 oral vaccine with or without the known adjuvant, a heat-labile lymphotoxin T subunit (LTB).

2. Materials and methods

2.1. Immunization protocols

LacE7 was provided from BioLeaders Corp. (Korea) and GENOLAC BL Corp. (Japan). LacE7 was generated from the recombinant *Lactobacillus casei* expressing HPV16 mutated E7 as previously described [18] and attenuated using heat. The attenuated *L. casei* were purified by washing several times with distilled water then dried to powder. LacE7 was insoluble in water-based solvents. Six-week-old female SPF C57BL/6 mice (CLEA Japan Inc., Japan) were used for immunization experiments. 1.0 mg/head of LacE7 were administered four times at weeks 1, 2, 4, and 6. All inoculums were suspended in PBS (200 μL/head) and administered once per day for five days each week via an intra-gastric tube after 3 h of fasting.

The Japanese herbal medicines, JTT or HET (40 mg/head/day, gifted from Dr. Keiichi Koizumi, University of Toyama) were mixed with powdered foods (5 g/head/day) which were taken consumed completely by five mice in a single cage. JTT or HET was administered to mice every day during each of the four rounds of LacE7 administration (weeks 0–6). Heat-labile *Escherichia coli* lymphotoxin, B subunit (LTB: 10 μg/head) was added to each LacE7 inoculum and administrated orally on the third day of each round of vaccination.

2.2. Sample collection

Lymphocytes, serum and intestinal washes were collected from immunized mice one week after the last inoculation (at week 7). After sacrifice, intestine, spleen and peripheral blood were obtained from five mice. Spleens were washed 3 times in HBSS. For intestinal specimens, the inside of intestinal tract was washed with 10 mL of HBSS with protease inhibitors after feces removal. The collected sera and intestinal washes were stored at −80 °C until use.

2.3. Preparation of murine splenocytes and intestinal mucosal lymphocytes

The intestines were opened longitudinally and shaken vigorously in RPMI1640 containing 10% FBS, 100 units/mL of penicillin and 100 μg/mL of streptomycin for 30 min at 37 °C. The resulting cell suspensions were passed through a BD Falcon Cell-strainer (BD Bioscience, USA) to remove tissue debris and were subjected to discontinuous density gradient centrifugation in a 15 mL tube layered from the bottom with 70% and 40% Percoll PLUS (GE Healthcare UK Ltd., England). The interface between the 70% and 40% layers contained lymphocytes with a cell viability of more than 95%. Splenocytes were prepared by gently teasing the spleen in PBS. Clumped debris was removed by centrifugation. Approximately $5–10 \times 10^6$ intestinal mucosal lymphocytes and 10^7 splenocytes were obtained from individual mice.

2.4. ELISPOT assays

50 μL of intestinal mucosal lymphocytes or splenocytes (5×10^6 cells/mL) were incubated for 24 h at 37 °C with antigen presenting cells comprised of 50 μL of splenocytes (5×10^6 cells/mL) treated with mitomycin C (75 μg/mL, Sigma, USA), and washed three times with PBS. 10 μL of synthetic peptide (working conc. = 1 μg/mL) corresponding to amino acids 49–57 of HPV16 E7 (a reported CTL epitope for C57BL/6 mice), mitogen (PMA 40 ng/mL + ionomycine 4 μg/mL), or medium alone (negative control) were added to a 96-well ELIIP plate (Millipore, USA) coated with anti-mouse IFNγ monoclonal antibodies from the Mouse IFNγ Kit (MABTECH AB, Sweden). IFNγ spot numbers were analyzed with a fully automated computer assisted video imaging analysis system, KS ELISPOT (Carl Zeiss Vision, Germany).

2.5. Cytokine measurements

Intestinal washes obtained from five mice were pooled and cytokine concentrations measured using the mouse Th1/Th2 ELISA Ready SET Go Kit (BD Bioscience, San Diego, CA, USA), which include IFNγ and IL-2 as representative Th1-type cytokines. The cytokine levels in each sample were normalized by total protein concentration. Measurements were repeated at least three times.

2.6. Statistical analysis

ELISPOT and ELISA data were presented as means ± standard deviations. Measurements and relative rates were compared between the immunization groups (5 mice/each group) using non-paired, two tailed Student's *t*-tests. A *p*-value of <0.05 was considered to be significant.

3. Results

3.1. The adjuvant effect of Japanese herbal medicines on E7-specific type 1 T cell responses

To examine the effect of oral administration of LacE7 vaccine plus Japanese herbal medicines on E7-specific type 1 T cell responses, the number of IFNγ-producing cells among mucosal lymphocytes or splenocytes was assessed by ELISPOT assay (Fig. 1). Each group of five mice was administered LacE7 (1.0 mg/head) orally or LacE7 plus JTT or HET (40 mg/head). JTT and HET were

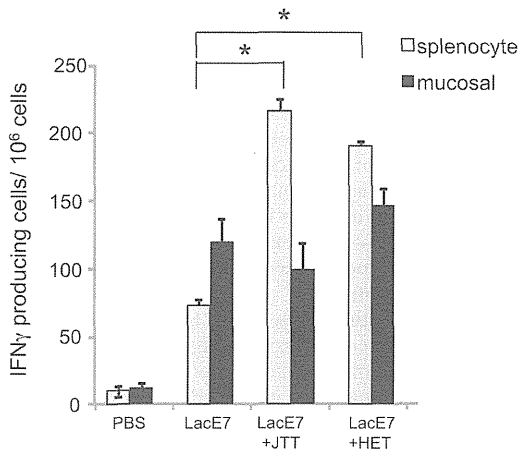

Fig. 1. Adjuvant effects of Japanese herbal medicines on type 1 T cell responses in mice orally immunized with Lac E7. The number of E7-specific IFNγ-producing cells among intestinal mucosal lymphocytes and splenocytes were assessed using ELISPOT assay. Five mice per group were immunized with LacE7 (1.0 mg/head) or PBS four times at weeks 1, 2, 4, and 6. JTT or HET was administered to mice every day during the four rounds of LacE7 administration. Mucosal lymphocyte and splenocytes were collected from immunized mice one week after last inoculation (at week 7) and approximately 10^5 of each type of lymphocyte were stimulated with the E7 peptide corresponding to HPV16E7 49–57 aa. Mean values with standard deviations are presented. Asterisks indicate those comparisons with statistical significance ($p < 0.05$) ($n = 5$).

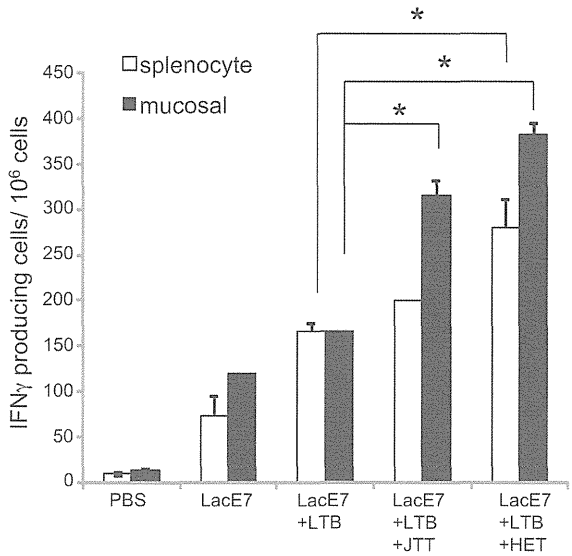

Fig. 2. Synergistic adjuvant effect of Japanese herbal medicines and LTB on type 1 T cell response. LTB (10 μg/head) was added to each LacE7 inoculum and administrated orally on the third day of each round of vaccination. This was performed in mice contemporaneously exposed to JTT, HET or control (no exposure). The number of E7-specific IFNγ producing cells among the collected intestinal mucosal lymphocytes and splenocytes was assessed using the ELISPOT assay as shown in Fig. 1. Mean values with standard deviations are presented. Asterisks indicate those comparisons with statistical significance ($p < 0.05$) ($n = 5$).

administered to mice as supplements to powdered food every day during four rounds of the LacE7 oral immunization. To detect potential adjuvant effects of the supplements on mucosal and systemic immunity, intestinal mucosal lymphocytes and splenocytes were collected from each mouse one week after the last immunization. The numbers of E7-specific IFNγ-producing cells among both mucosal lymphocytes and splenocytes increased significantly in LacE7-immunized mice but not in non-immunized (PBS) mice (Fig. 1). Oral immunization with LacE7 elicited a predominant mucosal E7-specific type 1 T cell response with E7-specific IFNγ-producing cell levels approximately 1.5–2.0-fold higher than those among splenocytes. Administration of LacE7 plus JTT or HET significantly improved systemic E7-specific type 1 T cell responses in splenocytes. However, neither JTT nor HET exhibited significant adjuvant effects on mucosal type 1 T cell responses (Fig. 1).

3.2. Adjuvant effects of the Japanese herbal medicines when combined with LTB on mucosal immune responses

Our initial data suggested that the use of additional adjuvants might be necessary to improve the mucosal cellular immune response to E7. We therefore repeated our investigations, adding oral LTB to LacE7 with each round of LacE7 oral immunization. Although the levels of E7-specific type 1 T cell response in mice given LacE7 plus LTB tended to increase, no significant differences were noted when comparing LacE7/LTB to LacE7 alone (Fig. 2). Mice exposed to either JTT or HET together with LTB and-LacE7 had improved mucosal E7-specific type 1 T cell response with approximately 2–2.5-fold higher levels of E7-specific mucosal IFNγ-producing cells when compared with sole exposure to LacE7 plus LTB (Fig. 2). Comparing Figs. 1 and 2, we noted that the addition of LTB to LacE7 plus either JTT or HET doubled the number of the IFNγ-producing cells among mucosal T cells, but not splenocytes. These data indicated that LTB and the Japanese herbal medicines act synergistically on the mucosal type 1 T cell response elicited by LacE7.

3.3. Local cytokine production induced by oral immunization with LacE7, LTB and Japanese herbal medicines

To confirm the characteristics of local cellular T cell responses stimulated by oral immunization, type 1 cytokine secretions were measured in the mucosal compartment. Levels of IFNγ and IL-2 production in intestinal washes obtained from immunized mice were measured by ELISA (Figs. 3 and 4). Both IFNγ and IL-2 levels in the mucosal fluid increased significantly in mice immunized orally with LacE7 when compared with non-immunized mice (PBS), consistent with a previous data that mucosal administration of *L. casei* alone induces Th1 cytokine production in a mucosal compartment [30]. Using comparisons mimicking those in Fig. 2, LacE7 plus either JTT or HET and LTB promoted secretion of both IFNγ and IL-2 into the intestinal lumen (Figs. 3 and 4). The secretion levels were 6–8-fold higher for IFNγ (Fig. 3) and 2–4-fold higher for IL-2 (Fig. 4) when compared with LacE7 alone. Administration of LacE7 plus LTB did stimulate increased cytokine secretion when compared with LacE7 alone. These results confirm that JTT or HET have synergistic effects when added to LacE7/LTB oral immunization protocols on local Th1 cytokine secretion, as well as the induction of E7-specific IFNγ-producing cells.

4. Discussion

The therapeutic HPV vaccines tested to date can induce enhanced cellular immune responses but none have demonstrated clinical efficacy against CIN [31–33]. We hypothesize that by using intramuscular or subcutaneous injection strategies, these approaches promote systemic cellular immunity, but not local mucosal immunity. Intraepithelial lymphocytes (IELs) residing in the cervical mucosa are most likely to represent the central effector cells for elimination of CIN and systemic vaccination with HPV E7 is not thought to elicit and retain enough E7-specific CTL within the cervical mucosa to eliminate CIN. We have previously observed

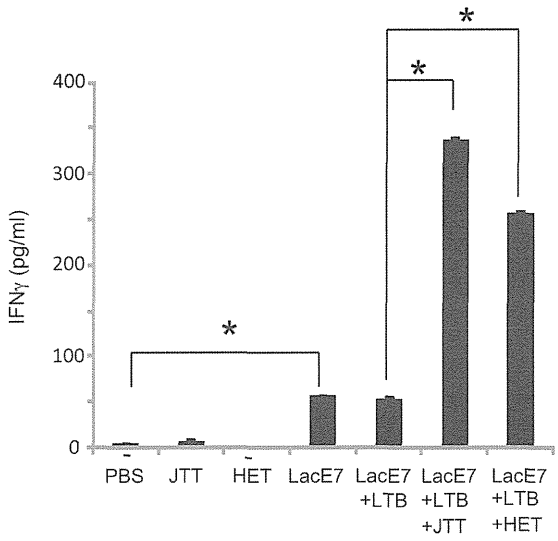

Fig. 3. IFNγ secretion into the intestinal compartment after immunization with LacE7 plus JTT or HET and LTB. IFNγ levels in the intestinal washes were measured by ELISA. The intestinal washes were collected at the same time points that were assessed in Fig. 1. Cytokine levels in each sample were normalized to corresponding total protein concentrations. Mean values with standard deviations are presented. The asterisks indicate those comparisons with statistical significance ($p < 0.05$) ($n = 5$).

and reported the induction of integrin $\alpha 4\beta 7^+$ mucosal T cells that provide E7-specific type 1 T cell responses after oral administration of LacE7 to mice [18]. We have also demonstrated that 25–30% of the $CD3^+$ cervical lymphocytes are integrin $\beta 7^+$ T cells [34]. In

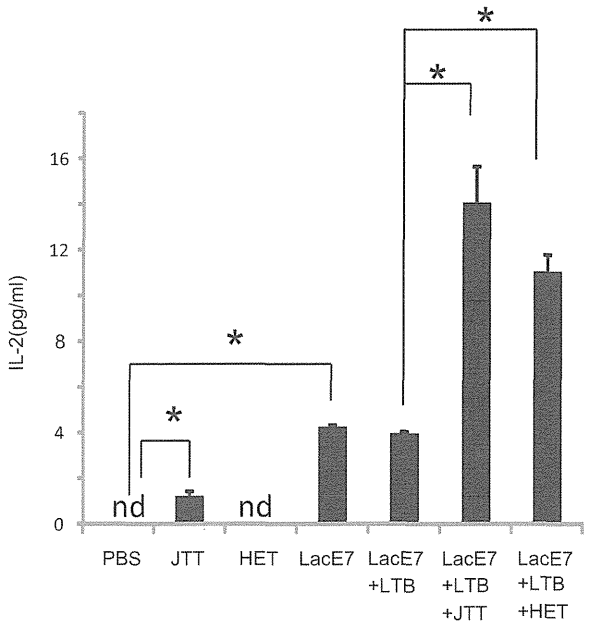

Fig. 4. IL-2 secretion into the intestinal compartment after immunization with LacE7 plus Japanese herbal medicine and LTB. IL-2 levels in the intestinal washes were measured by ELISA. The intestinal washes were collected at the same time points that were assessed in Fig. 1. Cytokine levels in each sample were normalized to corresponding total protein concentrations. Mean values with standard deviations are presented. The asterisks indicate those comparisons with statistical significance ($p < 0.05$) ($n = 5$).

our previous data, the number of vaccine induced E7-specific type1 T cells peaked at exposure levels of 1.0 mg/head and decreased with doses over 3.0 mg/head when mice were orally immunized with various doses of LacE7 (0.3–100 mg/head). We believe that 1.0 mg/head may be the optimal dose of LacE7 for induction of mucosal E7-specific type 1 T cells, because high-dose antigen may induce development of E7-specific regulatory T cells. These limitations led us to consider that the addition of an effective adjuvant agent might be more effective in improving E7-specific Th1 type responses than dose-escalation of LacE7. We chose to focus on two Japanese herbal medicines that have been reported to exhibit immunomodulatory effects.

Our data indicate that while JTT or HET alone exerts adjuvant effects on systemic but not mucosal type 1 T cell responses to LacE7, a combination of the mucosal adjuvant (LTB) with either Japanese herbal medicine dramatically improved the desired mucosal E7-specific type 1 T cell responses. These Japanese herbal medicine, when added to a conventional mucosal adjuvant, such as LTB, appear to act synergistically on mucosal vaccine-induced immune responses. The demonstrated adjuvant effects on mucosal immune response may be partially attributed to the strategy involving oral immunization of *L. casei*, which acts as an efficient vaccine carrier that delivers antigen across the gut to GALT but also exhibits its own vaccine adjuvant activities that promote type 1 T cell responses [4,35]. *Lactobacillus* species promote this type 1 T cell response polarization through interactions with dendritic cells (DCs) [36]. *Lactobacillus* activate DCs through TLR-2 and the activated DCs stimulate the proliferation of autologous $CD4^+$ and $CD8^+$ T cells and their secretion of IFNγ [37]. Recombinant *L. casei* alone can induce IFNγ production at mucosal sites [35]. Taken together, *L. casei* appears to be an excellent antigen delivery vehicle when mucosal type 1 T cell responses to vaccine antigen are desired. In our study, the levels of type 1 T cell responses to E7 barely increased in mice immunized with LacE7 and LTB when compared with LacE7 alone. However, the addition of Japanese herbal medicines to LacE7 and LTB resulted in two to three-fold higher levels of type 1 mucosal T cell responses when compared to LacE7 and LTB. In summary, the Japanese herbal medicines, JTT and HET act in synergy with *L. casei* and LTB in mucosal antigen delivery strategies. When Th1-type local T cell responses to vaccine antigen are desired, the combination of a Japanese herbal medicine and LTB promote efficient and mucosa-specific adjuvant activities when added to Lactobacillus delivery systems.

More specifically, the addition of specifically, the addition of specific Japanese herbal medicines and mucosal adjuvant to LacE7 may be an outstanding approach to generate E7-specific mucosal cytotoxic immune responses to HPV-associated neoplastic lesions.

Acknowledgements

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The Prevalence of Cervical Regulatory T Cells in HPV-Related Cervical Intraepithelial Neoplasia (CIN) Correlates Inversely with Spontaneous Regression of CIN

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Keywords
CD4+CD25+Foxp3+ regulatory T cells, cervical intraepithelial neoplasia, cervical lymphocytes, programmed cell death-1

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Problem
Local adaptive cervical regulatory T cells (Tregs) are the most likely direct suppressors of the immune eradication of cervical intraepithelial lesion (CIN). PD-1 expression on T cells induces Tregs. No studies have quantitatively analyzed the Tregs and PD-1+ cells residing in CIN lesions.

Method of study
Cervical lymphocytes were collected using cytobrushes from CIN patients and analyzed by FACS analysis. Comparisons were made between populations of cervical Tregs and PD-1+ CD4+ T cells in CIN regressors and non-regressors.

Results
A median of 11% of cervical CD4+ T cells were Tregs, while a median of 30% were PD-1+ cells. The proportions of cervical CD4+ T cells that were Tregs and/or PD-1+ cells were significantly lower in CIN regressors when compared with non-regressors.

Conclusions
The prevalence of cervical tolerogenic T cells correlates inversely with spontaneous regression of CIN. Cervical Tregs may play an important role in HPV-related neoplastic immunoevasion.

Introduction

HPV infection is a major cause of cervical cancer and its precursor lesion, cervical intraepithelial neoplasia (CIN). Natural history studies of CIN[1,2] show that most infections and most CIN lesions resolve spontaneously; only a minority persists and progress to cervical cancer. Studies showing that HIV-infected women and patients who are under treatment with immunosuppressive agents have an increased incidence of CIN lesions[3,4] suggest that cell-mediated immune response against HPV viral protein is important in the control of HPV infection and progression to CIN. We have previously reported that the presence of gut-derived effector lymphocytes within the cervix plays an important role in local cell-mediated

immune responses and correlates with CIN regression.[5] The presence of robust local tolerogenic cervical T-cell responses to HPV-related neoplastic lesions would be predicted to attenuate the effects of these local effector responses. We hypothesized that the proportion of tolerogenic lymphocytes among the CD4+ T cells in the cervix would decrease among women experiencing CIN regression, thereby allowing full effect of the changes previously seen among local effector cells.

It has been reported that CD4+CD25+Foxp3+ regulatory T cells (Tregs) play an important role in tumor-associated immunoevasion in cancers (ovarian, uterine cervical, endometrial, lung, breast, pancreas, renal cell, and thyroid cancers) as well as in other proliferative disorders such as melanoma and hepatoma.[6–15] Mechanisms underlying Treg suppressive functions have been abundantly reported. The high expression of CD25 (IL-2R) on Tregs has been thought to result in cytokine deprivation-induced apoptosis of effector T cells.[16] IL-10, TGF-β, and IL-35 are also important mediators of Treg suppressive function.[16] Tregs have been reported to suppress T effectors by ligating T-effector-expressed CD80, thereby inhibiting T-cell proliferation and cytokine production. Tregs kill effector T cells, other antigen-presenting cells, and NK cells in a manner dependent on granzyme and perforin.[16]

Natural Treg cells (nTregs) differentiate in the thymus and migrate to peripheral tissues while adaptive/induced Treg cells (iTregs) differentiate in secondary lymphoid organs and tissues including mucosa-associated lymphoid tissues (MALT).[17] iTregs play essential roles in mucosal tolerance, in the control of severe chronic allergic inflammation, in the prevention of parasite and other microorganism clearance, and in the obstruction of tumor immunosurveillance while nTregs have roles in preventing autoimmunity and preventing exaggerated immune responses. iTregs appear in the mesenteric lymph nodes during induction of oral tolerance, differentiate in the lamina propria of the gut in response to microbial signals, and are generated in chronically inflamed tissues. At a minimum, Foxp3+ iTreg development requires TCR stimulation and the cytokines TGF-β and IL-2. Integrin αEβ7+ dendritic cells (DCs) residing in the MALT produce both TGF-β and retinoic acid (RA), which mediate the differentiation of naïve T cells into Foxp3+ iTregs.[17]

The programmed cell death-1 (PD-1) and PD-ligand (PD-L) pathway is also critical in the suppression of immune responses. PD-1 is a molecule inducibly expressed on peripheral CD4+ and CD8+ T cells, NKT cells, B cells, monocytes, and some DC subsets when these cells are activated by antigen receptor signaling and cytokines.[16] nTregs and iTregs can express PD-1 and PD-L1, and the expression of ligand and receptor on the same cell conveys interesting implications. Engagement of PD-1 by its ligands during T-cell receptor (TCR) signaling results in two possible T-cell responses: 1) a diminution in T-effector responses and 2) an augmentation in differentiation of naïve T cells into Foxp3+ iTreg in a TGF-β-dependent manner.[16] There are synergistic effects between the PD-1/PD-L1 pathway and TGF-β in promoting Treg development. PD-L1 is expressed on a wide variety of tumors, and high levels of PD-L1 expression strongly correlate with unfavorable prognosis in a number of cancers.[18] To this point, ligation of PD-1 may induce and maintain iTregs within the tumor microenvironment, enhance the suppression of anti-tumor T-cell responses, and thereby allow tumor progression.

Several previous studies have shown that the prevalence of Tregs among PBMCs increases in CIN patients when compared with healthy controls.[19,20] These studies assess populations of circulating Tregs using flow cytometry. Characterization of the local lymphocytes residing in cervical lesions should better reflect local immune responses to pathogen. While Nakamura et al.[21] used Foxp3 immunostaining of human CIN lesions to report the number of local Foxp3+ cells residing in the CIN lesions by immunostaining of the tissues for Foxp3 and report that the number of Foxp3-immunoreactive cells is higher in CIN3 lesions than normal or CIN1-2 lesions, no studies have quantitatively assessed populations of local Tregs, likely iTregs, in the CIN lesions using flow cytometry. Possible associations between iTregs and the natural course of CIN have also never been studied.

We have previously characterized cervical lymphocytes collected from CIN lesions using a cytobrush and have demonstrated that the majority of cervical lymphocytes in these lesions are CD3+ T cells (median 74%) and that half of the cervical CD3 + T cells are CD4+ (median 54%).[5] In the present investigations, we have analyzed the relative proportions of two tolerogenic T-cell subsets, CD25+Foxp3+ Tregs and PD-1+ T cells, among cervical CD4+ T cells collected from CIN lesions. To determine whether there was a correlation between the frequency of cervical tolerogenic T cell and the natural course of

CIN, comparisons were made between tolerogenic T-cell subsets in the lesions of CIN regressors and non-regressors.

Materials and methods

Study Population

Cervical cell samples were collected using a cytobrush from 24 patients under observation after being diagnosed with CIN by colposcopically directed biopsy. All women gave written informed consent, and the Research Ethics Committee of the University of Tokyo approved all aspects of the study. Patients with known, symptomatic or macroscopically visible vaginal inflammation, or sexually transmitted infections were excluded from our study. To study the association between cervical tolerogenic lymphocytes and CIN progression, CIN patients with regression of cervical cytology (cases) were matched with control patients who did not exhibit cytologic regression over the same time period (measured from initial detection of abnormal cytology). In this study, cytological regression was defined as normal cytology at two or more consecutive evaluations conducted at 3–4 months intervals. For the comparison of CD4+CD25+Foxp3 Tregs and PD1+CD4+ cells, 12 patients were enrolled in the regression group, and the median follow-up duration was 16.5 (8–33) months. Twelve pairs of follow-up time-matched patients with persistent cytological abnormalities were enrolled in the non-regression group, and the median follow-up time was 19 (9–34) months. Patients were interviewed about their smoking history and their last menstrual period.

Collection and Processing of Cervical Lymphocytes

Cervical cells were collected using a Digene cytobrush as described previously.[5] The cytobrush was inserted into the cervical os and rotated several times. The cytobrush was immediately placed in a 15-mL tube containing R10 media (RPMI-1640 medium, supplemented with 10% fetal calf serum, 100 mg/mL streptomycin, and 2.5 μg/mL amphotericin B) and an anticoagulant (0.1 IU/mL of heparin and 8 nM EDTA). After incubating the sample with 5 mM DL-dithiothreitol at 37 °C for 15 min with shaking, the cytobrush was removed. The tube was then centrifuged at 330 *g* for 4 min. The resulting pellet was resuspended in 10 mL of 40% Percoll. This mixture was layered onto 70% Percoll and centrifuged at 480 *g* for 18 min. The mononuclear cells at the Percoll interface were removed and washed with PBS. Cell viability was greater than 95%, as confirmed by trypan blue exclusion, and fresh samples were immediately used for further analyses.

Immunolabeling and Flow Cytometry

Cervical immune cell preparations were immunolabeled with fluorochrome-conjugated mouse monoclonal antibodies specific for the following human leukocyte surface antigens: a programmed death-1 marker (FITC–anti-PD-1), a phycoerythrin cyanine 5.5 (PC5.5)-conjugated helper T-cell marker (PC5.5–anti-CD4), and an allophycocyanin (APC)-conjugated IL-2 receptor marker (APC–anti-CD25). After exposure to primary surface-labeling antibodies, cells were washed twice with FACS buffer (10% fetal calf serum, 1 mM EDTA, 10 mM NaN3), permeabilized with Foxp3 Fixation/Permeabilization working solution (eBioscience, San Diego, CA, USA), and immunolabeled with the anti-intracellular antigen antibody, phycoerythrin (PE)-conjugated anti-Foxp3 marker (PE–anti-Foxp3). Cells were then washed twice with Flow Cytometry Staining Buffer (eBioscience) and resuspended in Flow Cytometry Staining Buffer. Additional aliquots of the cell preparations were labeled in parallel with appropriate isotype control antibodies. Antibodies were purchased from eBioscience and BD (Franklin Lakes, NJ, USA). Data were acquired using four-color flow cytometry on FACSCalibar (Becton-Dickinson, Texarkana, TX, USA). A minimum of 5000 CD4+ T cells was analyzed per sample. The position of CD4+ T cells was determined by CD4 vs SSC gating. We used KALUZA® Flow Analysis Software (Becman Coulter, Brea, CA, USA) for data analysis.

HPV Genotyping

DNA was extracted from cervical smear samples using the DNeasy Blood Mini Kit (Qiagen, Crawley, UK). HPV genotyping was performed using the PGMY-CHUV assay method.[22] Briefly, standard PCR was conducted using the PGMY09/11 L1 consensus primer set and human leukocyte antigen-DQ (HLA-DQ) primer sets. Reverse blotting hybridization was performed. Heat-denatured PCR amplicons were hybridized to specific probes for 32 HPV genotypes

and HLA-DQ reference samples. The virological background (HPV genotyping) of 24 patients in our study is shown in Table I. HPVs 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 68, 73, and 82 were defined as high-risk HPVs according to an International Agency for Research on Cancer (IARC) multicenter study.[23]

Statistical Analysis

Statistical analyses, including calculation of medians and interquartile ranges (IQRs), were performed using the commercial statistical software package JMP® (SAS, Cary, NC, USA). Wilcoxon rank sum tests or Fisher's exact tests were applied for matched pair comparisons. *P*-values ≤ 0.05 were considered significant.

Results

Isolation of Cervical Tolerogenic T-cell Subsets in CIN Lesions

To assess cervical tolerogenic T cells, cervical samples were collected from CIN lesions positive for any HPV genotype and fractionated over a discontinuous Percoll density gradient to remove cervical epithelial cells. Cervical lymphocytes were then isolated from the interphase between Percoll and culture medium.[5] Cervical CD4+ T cells were identified among the isolated lymphocytes using CD4 vs SSC gating. The percentages of CD4+ cervical T cells that were CD25+Foxp3+ Tregs or that were PD-1+ were determined by flow cytometry. Two representative cases are displayed in Fig. 1(a,b), respectively. The proportion of cervical CD4+ T cells that were CD25+Foxp3+ was 14.2% whereas the proportion of CD4+ T cells that displayed PD-1 was 33.6% (bold lines). Among all CIN patients, a median of 11.7% (IQR: 7.3–14.6, $n = 24$) of CD4+ cervical T cells were CD25+Foxp3+ Tregs, while a median of 30.7% (20.2–38.5, $n = 24$) of CD4+ cells expressed PD-1. The proportions of tolerogenic T-cell subsets found in cervical preparations were markedly higher than those reported in circulating peripheral blood where approximately 5% of PBMCs are CD25+Foxp3+ Tregs[24] and 5% of peripheral CD4+ T cells are PD-1+.[25] These data indicate that the cervical mucosal T cells separation technique used for these investigations isolated a population of T cells with characteristics that suggest little to no contamination by peripheral blood. Further, should small amounts of contamination occur during isolation the effect on overall results would be predicted to be minimal.

Table I Patients infected with multiple HPV types were included.

HPV type	Total numbers (%)
16	5 (16.6)
18	2 (6.6)
31	1 (3.3)
45	1 (3.3)
51	1 (3.3)
52	3 (10)
53	3 (10)
55	3 (10)
56	4 (13.3)
58	5 (16.6)
70	2 (6.6)
Total	30 (100)

Of 24 patients, 4 (16.6%) were infected with multiple types. HPVs 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 68, 73, and 82 were defined as high-risk HPVs.

Correlation of Cervical Tregs and PD-1+ CD4+ cells in CIN Lesions with Menstrual Phase, HPV Types, Smoking History, and CIN Course

Many factors, including HPV genotypes, smoking, and other microbial infections, have been reported to associate with spontaneous regression or progression of CIN.[26] In this study, we obtained cervical Tregs from histologically diagnosed CIN patients and sought correlations between cervical Tregs and potential clinical factors, which may associate with the natural course of CIN. Patients with known, symptomatic or macroscopically visible vaginal inflammation, or sexually transmitted infections other than HPV were excluded from our study. All patients were diagnosed with CIN1-2 at the time of enrollment and followed with colposcopy and cervical cytology smears every 4 months.

To account for possible confounding factors, samples from our 24 CIN patients were reanalyzed after segregation by each of the following characteristics: menstrual phase (proliferative vs secretory), HPV genotype (high risk vs low risk), and smoking history (smoking vs non-smoking). The prevalence of CD25+Foxp3+ Tregs and of PD-1+ T cells among cervical CD4+ cells was compared between each of the

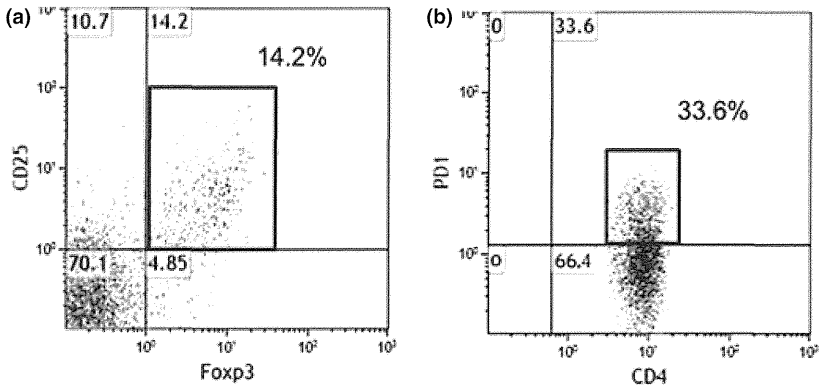

Fig. 1 Representatives of flow cytometric analysis of immune cells isolated from cervical intraepithelial neoplasia lesions. Bold lines delimit cervical CD4+CD25+Foxp3+ Tregs (a) and PD1+ CD4+ T cells (b). The indicated percentages represent percentage of total CD4+ T cells.

two groups using Wilcoxon rank sum testing (Table II). None of these possible confounders correlated with CD25+Foxp3+ Tregs and PD-1+ T cells results in CIN lesions, indicating that the tolerogenic T cells residing in the cervical mucosa were not influenced by smoking, hormonal status, or infecting HPV subtypes.

Next, we compared populations of CD25+Foxp3+ Tregs and PD-1+ T cells residing in the CIN lesions of regressors (n = 12) and non-regressors (n = 12) to determine whether there was an association between the frequency of cervical tolerogenic T-cell subsets and spontaneous regression of CIN. Twelve patients had spontaneous regression of their CIN lesions, and these women had a median follow-up duration of 16.5 (8–33) months. The non-regression group consisted of twelve women with persistent cytological abnormalities who were matched to the spontaneous regressor cohort by follow-up time. No significant differences were seen in the detection rates of high-risk HPV (58.3% vs 83.3%, P = 0.37), percent of CIN 2 at the enrollment (33.3% vs 58.3%, P = 0.4), and the median ages (33 years old vs 36, P = 0.44) of patients in the regression and non-regression groups. Among regressors, cervical CD25+Foxp3+ Tregs comprised a median of 7.3% (IQR: 6.3–11.4) of cervical CD4+ cells; the rate among non-regressors was 13.9% (IQR: 11.6–16.9). The frequency of cervical CD25+Foxp3+ Tregs in regressors was significantly lower than that in non-regressors (P = 0.0012) (Table II and Fig. 2). Similarly, cervical PD1+ CD4+ cells comprised a median of 20.8% (IQR: 15.8–31.9) of cervical CD4+ cells among regressors whereas a median of 35.1% (IQR:

Table II Correlation of the proportions of cervical Treg and PD-1+ cells among cervical CD4+ T-cell populations with clinical characteristics

		Percentage of total cervical CD4+ T cells			
Factors	Groups	CD25+Foxp3+ Tregs		PD-1+ cells	
Menstrual phase	Proliferative	10.26 (7.04–15.4)	P = 0.94	29.8 (22.7–39.5)	P = 0.72
	Secretory	12.0 (7.1–14.2)		28.1 (18.9–36.7)	
HPV genotype	High risk	11.8 (7.8–14.2)	P = 0.67	29.8 (20.3–38.2)	P = 0.82
	Low risk	7.4 (6.7–15.7)		33.5 (18.5–45.4)	
Smoking	Smoking	10.2 (7.3–14.7)	P = 0.73	29.8 (19.5–39.5)	P = 0.80
	Non-smoking	10.8 (5.0–15.9)		24.6 (19.6–40.9)	
CIN course	Regression	7.3 (6.3–11.4)	P = 0.0012	20.8 (15.8–31.9)	P = 0.018
	Non-regression	13.9 (11.6–16.9)		35.1 (30.2–42.6)	

Association of cervical CD4+CD25+Foxp3+ Tregs and PD1+CD4+ cells with menstrual cycle, HPV genotype, smoking, and cervical intraepithelial neoplasia (CIN) course were shown.

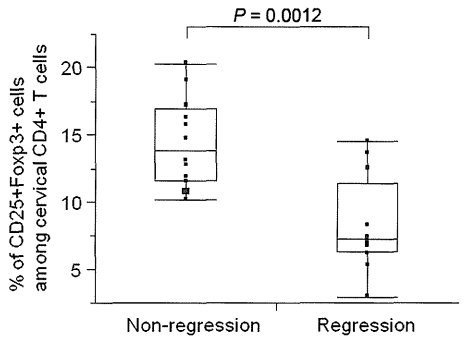

Fig. 2 Association of cervical Tregs with the natural course of cervical intraepithelial neoplasia. Among regressors, cervical Tregs comprised a median of 7.33% [Interquartile ranges (IQR): 6.38–11.4, n = 12] of CD4+ cervical T cells; the rate among non-regressors was 13.9% (IQR: 11.6–16.9, n = 12); P = 0.0012.

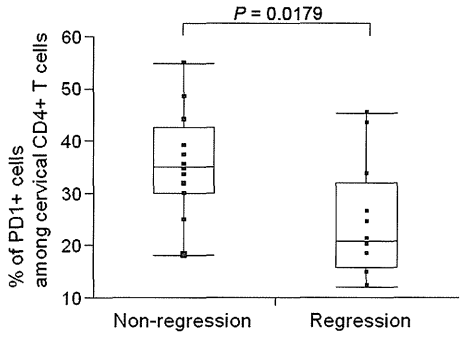

Fig. 3 Association of cervical PD-1+ CD4+ T cells with the natural course of cervical intraepithelial neoplasia. Among regressors, cervical PD1+ cells comprised a median of 20.8% [Interquartile ranges (IQR): 15.8–31.9, n = 12] of CD4+ cervical T cells; the rate among non-regressors was 35.1% (IQR: 30.2–42.6, n = 12); P = 0.0179.

30.2–42.6) among non-regressors. Again, the frequency of cervical PD-1+ CD4+ cells in regressors was significantly lower than that in non-regressors (P = 0.017) (Table II and Fig. 3).

Discussion

Although many studies have been reported about the positive association between tolerogenic lymphocytes and poor prognosis in many cancers, there are limited data on similar associations in women with HPV-related cervical precursor lesions. Our results show that the prevalence of CD25+ Foxp3+ Tregs and of PD1+ CD4+ T cells residing in cervical precursor lesions inversely correlates with spontaneous regression of CIN.

The peripheral population of Foxp3+ Tregs includes nTregs and iTregs. iTregs play essential roles in mucosal tolerance, in the control of severe chronic allergic inflammation, and in the prevention of organism clearance and tumor immunosurveillance, while nTregs have roles in preventing autoimmunity and exaggerated immune responses.[17] We would predict that the majority of cervical CD25+Foxp3+ Tregs assessed in this study are iTregs although definitive isolation of iTregs is hampered by the lack of suitable surface markers that distinguish iTreg and nTreg cell populations.

In this study, cervical Treg prevalence negatively correlated with regression of CIN (Fig. 2) but did not correlate with CIN grade (data not shown). Supporting our data, several previous studies have shown a positive correlation between Treg prevalence in peripheral blood and high grade of CIN.[19,20] Of course, cervical iTregs and circulating Tregs may differ in their TCR repertoire. iTregs are known to differentiate from mature naïve CD4+ cells through the effects of TGF-β and RA secreted by mucosa-associated DCs.[17] In our data, the proportion of CD25+Foxp3+ Tregs among total cervical CD4+ cells (a median of 11%) was twofold higher than previously reported peripheral blood levels (approximately 5%). This suggests that iTregs may be generated continuously, probably in an antigen-depending manner, and accumulate in chronically HPV-infected tissues and CIN lesions. Others have reported that Foxp3 mRNA levels in cervical samples that included exfoliated epithelial cells and cervical lymphocytes are higher among high-grade squamous intraepithelial lesion (HSIL) patients when compared with low-grade squamous intraepithelial lesion (LSIL) patients.[27] However, it is unknown whether Foxp3 mRNA levels in these cervical samples parallel the number of Tregs because cervical lymphocytes were not specifically isolated in this study.

Although the persistence of HPV infection was not followed in the present study, Molling et al.[20] reported that CD4+CD25hi Treg frequency correlates with persistence of HPV type 16. Tregs may inhibit the HPV clearance by immune cells such as invariant natural killer T cells.

TGF-β is critical to the induction and maintenance of Foxp3+ Tregs, with particular importance in the induction of iTregs from naive T cells and in the conversion of effector T cells to iTregs. Several studies have demonstrated that the expression of TGF-β and RA receptors in cervical specimens is lower in

CIN lesions when compared with normal epithelium.[28,29] In these studies, there was no correlation between TGF-β mRNA levels and either CIN grade or CIN natural course. TGF-β-induced iTreg frequency may be a more direct predictor of CIN progression than TGF-β. In fact, measurement of tolerogenic T-cell frequency in CIN lesions has the potential to prove useful in determining individualized screening and treatment paradigms.

Whether sex hormones modulate the prevalence and function of Tregs remains controversial. Arruvito et al. reported that the proportion of Foxp3+ cells within the peripheral blood CD4+ T-cell population increases during the late follicular phase when compared with the luteal phase.[29] The expansion of Tregs during the follicular phase was highly correlated with serum estradiol (E2) levels.[30] In contrast, Weinberg et al. reported recently that there are no significant correlations between changes in serum E2 levels and the prevalence of any circulating Treg subtypes or between changes in serum progesterone levels and the proportion of CD8+ Foxp3+ Tregs in peripheral blood samples.[31] The effect of smoking on the generation of tolerogenic T cells is also controversial.[32–34] Note that all of the above studies assess peripheral circulating rather than local cervical Tregs. Our data on the latter cells revealed no correlations between cervical Treg prevalence and either menstrual phase or smoking.

In this study, we focused on PD-1+ CD4+ T cells as well as Foxp3+ Tregs as engagement of PD-1 by its ligands on T cells is critical to the differentiation of naïve T cell into Foxp3+ iTregs. Furthermore, Tregs and the PD-1/PD-L pathway are integral in terminating immune responses and augmenting the suppression of anti-tumor T-cell responses. In short, the PD-1 pathway controls the development, maintenance, and function of iTregs at mucosal sites. Here, we show that PD-1+ T cells are more frequently found among cervical T cells than among PBMCs and that the prevalence of PD1+ T cells in CIN lesions (likely reflecting cervical iTregs) correlates inversely with spontaneous regression of CIN. Assessment for other tolerogenic T-cell subsets (e.g., Foxp3-IL10+ Tr1, Foxp3-TGF-β+ Th3) in this study, while potentially informative, was limited by the number of cervical lymphocytes that could be isolated from a single cytobrush sample.

In summary, even the study population is small and the results are limited, our flow cytometric analyses demonstrate for the first time that a prevalence of CD4+ CD25+ Foxp3+ Tregs infiltrating into CIN lesions significantly correlates with regression of CIN regardless of HPV subtype. Conversely, a high prevalence of lesional cervical Tregs may be responsible for CIN persistence as well as HPV infections and might function as a useful predictive biomarker for progression of CIN.

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ORIGINAL ARTICLE

Antimicrobial susceptibility and penicillin-binding protein 1 and 2 mutations in *Neisseria gonorrhoeae* isolated from male urethritis in Sapporo, Japan

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Abstract The spread of antimicrobial-resistant *Neisseria gonorrhoeae* worldwide is a critical issue in the control of sexually transmitted infections. The purpose of this study was to clarify recent trends in the susceptibility of *N. gonorrhoeae* to various antimicrobial agents and to compare these data with our previous data. Minimum inhibitory concentrations (MICs) of various antimicrobial agents were determined in *N. gonorrhoeae* strains clinically isolated from male gonococcal urethritis. In addition, amino acid sequencing of penicillin-binding protein (PBP) 2, encoded by the *penA* gene, was analyzed so that genetic analysis of mosaic PBP 2 could clarify the susceptibility of the strains to cefixime and other cephalosporins. The susceptibility rate for ceftriaxone, cefodizime, and spectinomycin, agents whose use is recommended by the guideline of the Japanese Society of Sexually Transmitted Infections (JSSTI), was 100 %. The susceptibility rates of the strains to penicillin G and ciprofloxacin were lower than those in previous reports. Mosaic PBP 2 structures were detected in 51.9 % of the strains and the MICs of the strains with the mosaic PBP 2 to cefixime were much higher than those of the strains without the mosaic PBP 2. In the clinical situation, the treatment regimen recommended by the JSSTI remains appropriate; however, the susceptibility to cephalosporins should be intensively surveyed because strains with mosaic PBP 2 were commonly detected.

Keywords *Neisseria gonorrhoeae* · Antimicrobial resistance · Penicillin-binding protein · Urethritis

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Introduction

The current problem with *Neisseria gonorrhoeae*, the pathogen of male gonococcal urethritis, is the spread of antimicrobial-resistant strains. Quinolone-resistant *N. gonorrhoeae* (QRNG), in particular, is widely spreading throughout many countries [1]. In addition, cefozopran-resistant *N. gonorrhoeae* (CZRNG), defined as strains resistant to almost all the cephalosporins except for ceftriaxone (CTRX) and cefodizime (CDZM) [2], has been observed, and the emergence of CTRX-resistant *N. gonorrhoeae* has been reported in Japan [3]. In recent years, strains with reduced susceptibility to cephalosporins, especially cefixime (CFIX), have been widely reported and such strains have been shown to possess mosaic penicillin-binding protein (PBP) 2 [4]. Therefore, it is critically important to survey the susceptibility to antimicrobial agents in clinical isolates of *N. gonorrhoeae* regionally when we determine whether the optimal treatment can be performed. In this study, we assessed the susceptibilities to several antimicrobial agents and analyzed the molecular mechanism of reduced susceptibility to CFIX in clinical isolates of *N. gonorrhoeae* in Sapporo, Japan.

Materials and methods

Bacterial strains

N. gonorrhoeae was isolated from male patients with gonococcal urethritis clinically diagnosed by expert urologists between January 2007 and January 2009. Pus discharged from the external urethral meatus was collected as the specimen. We obtained 52 clinical isolates from these patients. The specimens were inoculated on Thayer-Martin selective agar (Becton–Dickinson, Franklin Lakes, NJ, USA) and incubated at 35 °C for 24–48 h in a 5 % CO_2 atmosphere. *N. gonorrhoeae* was identified as a gram-negative diplococcus by positive oxidase reaction. Finally, to confirm the identification, *N. gonorrhoeae* was identified using Gonochek II (Cosmo Bio, Tokyo, Japan). Strains ATCC49226 and FA1090 (ATCC700825) were obtained from the American Type Culture Collection. ATCC49226 was used as a quality control for susceptibility testing and FA1090 was used as a reference strain for the genetic analysis.

Antimicrobial susceptibility test

The minimum inhibitory concentrations (MICs) of penicillin G (PCG), amoxicillin (AMPC), ceftriaxone (CTRX), cefodizime (CDZM), cefixime (CFIX), cefditoren (CDTR), spectinomycin (SPCM), azithromycin (AZM), tetracycline (TC), minocycline (MINO), ciprofloxacin (CPFX), levofloxacin (LVFX), prulifloxacin (PUFX), and fosfomycin (FOM) against clinically isolated *N. gonorrhoeae* were determined using GC agar base (Becton–Dickinson) with 1 % IsoVitalX enrichment (Becton–Dickinson) according to the Clinical and Laboratory Standards Institute (CLS) procedure [5]. We compared the antimicrobial susceptibilities to the above antimicrobial agents in clinical isolates with those found in our previous reports [6, 7].

We defined the MIC50 as the concentration at which 50 % of isolates were inhibited and the MIC90 as that at which 90 % of them were inhibited. The MIC breakpoints for PCG, CTRX, CFIX, SPCM, TC, and CPFX were defined according to the report of the CLSI [5]. Although the MIC breakpoints for CDZM, MINO, and LVFX were not defined in the report of the CLSI [5], they could be predicted from a previous report [8]. The susceptibility rate was defined as the proportion of strains with an MIC value of the breakpoint or less divided by the total number of strains.

Classification of PBP 1 and PBP 2

Full-length *ponA* and *penA* genes were amplified from genomic DNA by polymerase chain reaction (PCR). The primers used for PCR amplification, and the conditions of PCR and sequencing were reported in detail previously [9]. We especially investigated mosaic PBP 2 and analyzed the translated amino acid sequences of mosaic PBP 2 to compare them with those in previous reports. In the report by Takahata et al. [9], strains classified as group I had no alteration at any mosaic mutation in PBP 2 and no alteration at amino acid 421 in PBP 1. Takahata et al. [9] also classified the strains into subgroups Ia, Ib, Ic, and Id according to the pattern of the amino acid substitutions. Strains classified as group II had no alteration at a mosaic mutation in PBP 2 and possessed the L421P mutation in PBP 1; the group II strains were classified into subgroups IIa, IIb, IIc, and IId according to the pattern of the amino acid substitutions. Strains classified as group III had both the L421P mutation in PBP 1 and mosaic PBP 2, and were classified into subgroups IIIa, IIIb, IIIc, IIId, and IIIe. When any strains did not fit these classifications [9], we classified them into additional groups and/or subgroups. The mosaic-like structure of PBP 2 was classified into four types according to the above report by Takahata et al. [9]. Because the report by Takahata et al. [9] revealed that the amino acid substitutions in *N. gonorrhoeae* were responsible for its reduced susceptibility to CFIX, we analyzed the association between the pattern of the amino acid substitutions in each strain and the geometric mean MIC in each (sub)group. The geometric mean MIC indicates the typical value of a set of MICs in each group. The increases in the geometric mean MICs for the strains in each (sub)-group were calculated, considering the MIC in the group Ia strains to have the value of 1.

Results

Antimicrobial susceptibility

Antimicrobial sensitivity tests were done and MICs were determined in 51 of the 52 clinical isolates of *N. gonorrhoeae* because 1 isolate did not grow well enough to allow determination. The susceptibility rate to PCG was extremely low and that to quinolone was also low. However, the susceptibility rate to CFIX was 92.2 %. No strains resistant to CTRX or SPCM were found (Table 1).

The changes in MIC values and susceptibility rates in clinical isolates of *N. gonorrhoeae* obtained from 1998 [6], 2001 [7], and 2007–2009 showed decreased susceptibility to PCG and CPFX. The susceptibility to CFIX had decreased slightly. The susceptibility rate for CTRX and SPCM was 100 %; however, the values for the ranges of the MIC, MIC50, and MIC90 for SPCM have been increasing recently (Table 2). Although SPCM can still be recommended for the treatment of male gonococcal urethritis, strains with low susceptibility to this agent were predominant in the clinical isolates.