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Research Article

The Observation of Humoral Responses after Influenza Vaccination in Patients with Rheumatoid Arthritis Treated with Japanese Oriental (Kampo) Medicine: An Observational Study

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Objective. The efficacy of influenza vaccination in patients treated with Japanese Oriental (Kampo) Medicine is unknown. The objectives of this study were to observe the efficacy of influenza vaccination in RA patients treated with Kampo. *Methods.* Trivalent influenza subunit vaccine was administered to 45 RA patients who had received Kampo. They were divided into 2 groups: RA patients treated without MTX ("without MTX group") and treated with MTX ("with MTX group"). Antibody titers were measured before and 4 weeks after vaccination using hemagglutination inhibition assay. *Results.* Geometric mean titers (GMTs) of anti-influenza antibodies significantly increased for all influenza strains. Response to the influenza vaccination in RA patients treated with Kampo was not lower than that of healthy subjects and the response in the "with MTX group" had a tendency to be higher than that in RA patients treated with MTX in the previous study. There was no significant difference in the GMT after 4 weeks between the "with MTX group" and the "without MTX group." A decreased efficacy in both seroprotection and seroconversion was not found in the "with MTX group." *Conclusion.* These observations may open the way for further clinical trials to establish the efficacy for the influenza vaccination in RA patients treated with Kampo.

1. Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease that is associated with immunologic changes in T cells and B cells. In patients with RA, an impaired ability to react to antigens and an increased peripheral blood CD4/CD8 ratio has been observed in T cells [1, 2]. The presence of soluble interleukin-2 (IL-2) receptors in serum has showed T cell activation [2, 3]. Furthermore, T cell receptor rearrangement excision circles measured from T cells from RA patients were substantially lower than those in healthy controls, because the T cell receptor repertoire has been oligoclonal, which suggests on antigen selection and restriction of the repertoire [4]. There is also a decline in the thymic output of T cells. This premature aging of T cells in RA may have very severe effects on vaccine responses, which are well known to decrease with aging [5]. Additionally, the function of regulatory T cells (CD4+, CD25+) may be abnormal in active RA patients, with a lack of suppression of CD4+ or CD8+ T cells [6].

The multiple immunologic effects of the disease process may in part explain why patients with RA are considered immunocompromised and at increased risk of infection [7]. Therefore, although the exact prevalence, morbidity, and mortality of influenza in patients with RA are unknown, a yearly influenza vaccination is recommended [8]. The influenza vaccination is safe and results in protective levels of anti-influenza antibodies in most RA patients, even when they are treated with prednisone, disease-modifying antirheumatic drugs (DMARDs), or tumor necrosis factor-blocking agents [9, 10].

In Japan, Japanese traditional herbal (Kampo) Medicine, which is covered by national health insurance, is often

prescribed in the primary care field and is also applied as an alternative treatment for serious diseases such as RA. Since ancient times, many kinds of Kampo formulae have been used traditionally and are found to be clinically effective for RA treatment. These formulae usually contain components from several medicinal plants that are thought to exert anti-inflammation and immune-regulator effects and are effective for treating RA [11–13]. We have demonstrated that kampo formula possessed antirheumatic effects in vitro and in vivo [14, 15]. Furthermore, we have observed that the administration of kampo formula partially suppressed T cell activation in collagen induced arthritis (CIA) mice [16]. However, the effectiveness of the influenza vaccination in RA patients treated with Kampo remedy is still not known. The purpose of this study is to investigate the response to the influenza vaccination in RA patients treated with Kampo remedy.

2. Patients and Methods

2.1. Patient's Profile. Patients who visited our department in 2010-2011 had to fulfill the American College of Rheumatology 1987 revised criteria for the classification of RA and were selected in a random sampling method. All patients had been treated with Kampo formulae, which were often administered to the patients with RA.

2.2. Study Design. An observational study design was utilized in this study. Forty-five patients were entered into this design. Patients received the influenza vaccine intramuscularly from October 2010 until January 2011. Immediately before and 4 weeks after vaccination, blood was drawn for the measurement of C-reactive protein levels (CRP), erythrocyte sedimentation rate (ESR), and anti-influenza antibodies. The Disease Activity Score in 28 joints (DAS28) [17] was recorded before and 4 weeks after vaccination. Information on previous influenza vaccinations was obtained from all participants, and adverse effects occurring in the first 7 days postvaccination were recorded. This study was approved by the Ethics Committee of Gunma Central & General Hospital in Aug 2010.

2.3. Vaccine. We used a trivalent influenza subunit vaccine (2010-2011; Daiichi-Sankyo co.ltd Tokyo Japan) containing purified hemagglutinin and neuramidase of the following strains: A/California/7/2009 (H1N1)-like strain (A/H1N1 strain), A/Victoria/210/2009 (H3N2)-like strain (A/H3N2 strain), and B/Brisbane/60/2008-like strain (B strain).

2.4. Hemagglutination Inhibition Assay (HIA). The HIA was used for the detection of anti-influenza antibodies. HIAs were performed with guinea pig erythrocytes in accordance with standard procedures [18]. The following parameters for efficacy of the vaccination based on the anti-influenza antibody response were evaluated: geometric mean titer (GMT), fold increase in titer, 4-fold titer rise resulting in a postvaccination level of 40 (seroconversion), and titer rise to 40 $\geq$ (seroprotection). HIA titers 40 are generally considered to be protective in healthy adults [19].

3. Results

3.1. Patient Characteristics. Forty-five RA patients were administered Kampo treatment. They were divided into 2 groups as follows: 16 RA patients treated without MTX (without MTX group) and 23 RA patients treated with MTX (with MTX group). Patients treated with tacrolimus (TAC) or biologics were excluded from the patients in the without MTX group, and patients treated with biologics were excluded from the patients in both the with MTX and without MTX group. Their characteristics were shown in Table 1.

3.2. The Response to the Influenza Vaccination. Each GMT after 4 weeks vaccination was 78.8 $\pm$ 119.7, 35.7 $\pm$ 33.6, and 27.3 $\pm$ 27.3 in A/H1N1, A/H3N2, and B strain, respectively (Table 2). Response to the influenza vaccination in RA patients treated with Kampo formulae was not lower than that of healthy subjects in previous studies [20, 21]. There was no significant difference in the GMT after 4 weeks between the "with MTX group" and the "without MTX group." The GMT in the with MTX group was higher than in the without MTX group (Table 2). The response in the with MTX group had a tendency to be higher than that in RA patients treated with MTX in the previous study [21]. Furthermore, we calculated the fold increase as well as the GMT. The mean fold increase in each group was as follows: 6.5, 2.6, and 2.1, respectively (Table 2). The fold increase in the with MTX group also had a tendency to be higher than in the without MTX group, although this was not significant.

3.3. Seroprotection and Seroconversion. After 4 weeks vaccination, the percentage of patients who possessed the 40 $\geq$ titer in A/H1N1 was 53.3, 50.0, and 65.2% in total RA patients, without MTX group and with MTX group, respectively (Figure 1). There was no significant difference between the with MTX and the without MTX groups and a decreased efficacy in seroprotection was not found in the with MTX group. In A/H3N2, the percentage of patients who possessed the 40 $\geq$ titer was 46.7, 50.0, and 52.2%, and in the B strain, 28.9, 25.0, and 39.1% in total RA patients, without MTX group, and with MTX group, respectively. The seroprotection effect observed in the with MTX group had a tendency to be higher than results in the previous study [21]. In seroconversion, the percentage of patients who possessed 40 $\geq$ titer induced by 4-fold increase was 40.0, 35.6, and 15.6%, respectively (A/H1N1, A/H3N2, and B Strain). There was no significant difference between the with MTX and the without MTX groups also in seroconversion (data not shown).

3.4. The Influence of Influenza Vaccination upon RA Disease Activity. The DAS28 did not change after vaccination. There was no adverse reaction by influenza vaccination.

4. Discussion

Kampo medicine, which is covered by national health insurance in Japan, is often prescribed in the primary care field,

Table 1: Characteristics at baseline of RA patients in this study.

	Total	Without MTX group*	With MTX group**
Age, mean ± SD years	56.2 ± 13.5	58.6 ± 10.5	54.1 ± 12.6
No. (%) female/No. (%) male	42 (93)/3 (7)	15 (94)/1 (6)	22 (92)/2 (8)
Duration of RA mean ± SD years	12.2 ± 14.1	13.5 ± 15.6	10.9 ± 11.6
MTX dosage, mean ± mg/week	5.1 ± 3.8	0	7.6 ± 2.5
PSL dosage, mean ± SD mg/day	2.1 ± 2.0	1.6 ± 1.5	2.4 ± 1.9
Taking DMARDs, No.			
Bucillamine	1	1	0
Sulfasalazine	11	8	2
Tacllolimus	4	0	4
DAS28 CRP	3.2 ± 1.1	2.9 ± 1.0	3.3 ± 1.4

*Without MTX group: patients treated with classical DMARDs alone. Patients treated with tacllolimus were excluded. **with MTX group: patients treated with MTX, but not biologics.

Table 2: GMTs and fold increase in GMT for influenza A/H3N2, A/H1N1, and B strains in RA patients treated with Kampo formulae before and after administration of influenza vaccines.

	Total	Without MTX group*	With MTX group**
GMT, mean ± SD			
A/H1N1 strain			
Baseline	12.1 ± 14.0	11.0 ± 12.1	14.1 ± 15.0
4 weeks later	78.8 ± 119.7	39.6 ± 39.3	115.9 ± 148.8
A/H3N2 strain			
Baseline	13.5 ± 13.9	16.0 ± 19.7	11.7 ± 10.2
4 weeks later	35.7 ± 33.6	33.1 ± 21.8	39.1 ± 40.2
B strain			
Baseline	12.8 ± 10.3	13.9 ± 9.2	11.4 ± 11.5
4 weeks later	27.3 ± 27.8	22.8 ± 19.2	31.4 ± 34.0
Fold increase, mean (range)			
A/H1N1 strain	6.5 (1 to 64)	3.6 (1 to 16)	8.2 (1 to 64)
A/H3N2 strain	2.6 (1 to 16)	2.1 (1 to 8)	3.3 (1 to 16)
B strain	2.1 (1 to 16)	1.6 (1 to 4)	2.7 (1 to 16)

*Without MTX group: patients treated with classical DMARDs alone. Patients treated with tacllolimus were excluded. **with MTX group: patients treated with MTX, but not biologics.

and is also applied as an alternative remedy for RA. The efficacy for RA of Kampo medicines has been demonstrated by case or case series reports and several clinical trials. From these reports, the clinical effectiveness of Kampo therapy is almost similar to that of classical DMARDs, such as bucillamine (Bc) and salazosulfapyridine (SASP). Additionally, several investigators have demonstrated the immunomodulatory effects of Kampo medicine in RA patients as well as an arthritis mouse model, such as CIA [11, 12, 14]. We have also reported that Kampo therapy resulted in a decrease in serum IL-6 levels, but not TNF-α levels, as well as the suppression of arthritis development, based on the observations of the CIA mouse model [15]. Furthermore, it has been reported that Kampo medicine is probably effective against infection. The efficacy of Kampo therapy on atypical mycobacterium pneumonia and aspiration bacterial pneumonia has been demonstrated [22, 23], and these effects may be caused by immune-regulator effects, but not direct antibacterial effects. On the other hand, RA patients are susceptible to both viral and bacterial infections. In Japanese RA patients, major causes of death included malignancies (24.2%), respiratory involvement (24.2%) including pneumonia (12.1%) and interstitial lung disease (ILD) (11.1%), cerebrovascular disease (8.0%), and myocardial infarction (7.6%) [24]. Infectious disease is one of the critical factors in the mortality of RA patients. Therefore, a yearly influenza vaccination is recommended by the Center for Disease Control and Prevention (CDC) [25, 26]. However, the immune response to the influenza vaccination has not been reported in RA patients treated with Kampo medicine. This is the first report demonstrating the titer of anti-influenza antibodies before and after influenza vaccination in RA patients administered Kampo formulae.

The response to the influenza vaccination in our population was almost similar to previous results in healthy subjects. Kampo therapy may be beneficial for RA patients from the clinical viewpoint of protection against influenza

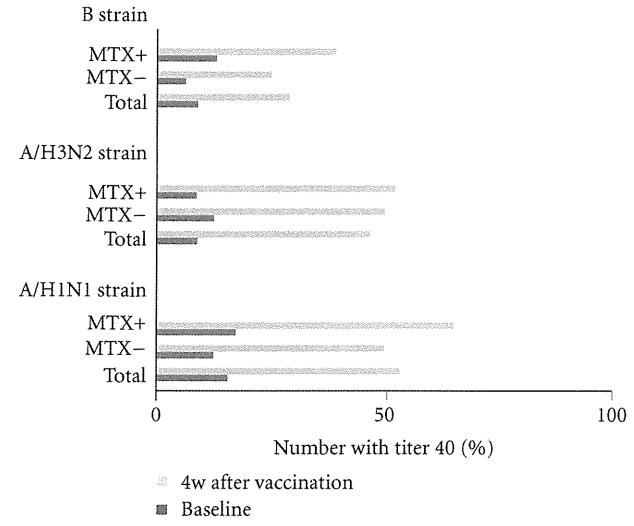

FIGURE 1: Percentage of patients with anti-influenza titers ≥40, as determined by a hemagglutination inhibition assay for each strain after vaccination with a trivalent influenza subunit vaccine, in total RA patients, RA patients treated with MTX, and RA patients treated without MTX. Solid bars represent prevaccination titer ≥40; open bars represent post vaccination titer ≥40.

virus infection as well as suppression of RA disease activity. However, there are various opinions about the efficacy of the influenza vaccination in RA patients. Some reports demonstrate both no differences and significant differences in the response rate between treatment with and without MTX in RA patients [20, 27–29]. This discrepancy may be caused by the different endpoints when measuring the response to the influenza vaccination and different influenza virus roots. Therefore, our data should be limited in reference to the adjuvant effects of Kampo therapy. However, as the baseline titers in this study were less than previous studies, we consider Kampo therapy to be partially beneficial for RA patients in seroprotection and seroconversion. In addition, it has been reported that the response to vaccination was significantly less in patients treated with anti-TNF-a and anti-CD20 antibody (rituximab) drugs than RA patients without biologics [21, 29]. We have checked the titers of the 5 patients treated with biologics, and they were less than those of other RA patient groups (data not shown). Kampo therapy may not influence the response to the influenza vaccination in RA patients treated with biologics. To analyze this problem, further clinical observational studies will be required using a large number of patients.

The RA disease activity by DAS28 did not change after vaccination in our patients. It is generally thought that the vaccination does not influence the disease activity and the titer of the serological markers. A recent report demonstrates that influenza vaccination did not alter the percentage of healthy adults with positive autoantibodies [30].

We have reported several patients with MTX-resistant RA as being successfully treated with Kampo medicine; however, it is still not clear as to how Kampo medicine acts on arthritis in humans [31]. We previously demonstrated that Kampo medicine suppressed polyclonal B cell activation, but not T cell activation, significantly in the CIA mouse model [14, 15]. Recently, it has been clarified that the development of arthritis in the CIA mouse contributed to the differentiation of IL-17 producing cells (Th17), dependent on IL-6 and TGF-β [32, 33]. In our previous study using CIA, Kampo medicine decreased the serum IL-6 levels, but not TNF-α, suggesting that the suppression of Th17 cell activation by Kampo therapy probably improved the development of arthritis. Thus, we suggest that Kampo medicines do not influence the function of antigen presentation in dendrite cells or macrophages. Based on these findings, we suggest that Kampo therapies do not suppress the response to the influenza vaccination in RA patients. Besides, in innate immunity, we have demonstrated that Juzentaihoto enhanced the production of iNOS in macrophages [34] and the upregulation of NK receptor's expression (Killer-cell immunoglobulin-like receptors) in NK cells [35]. Additionally, the direct anti-influenza virus actions of cinnamon cortex and ephedrae herba (the main herbs composing kampo formulae) have been demonstrated, while these actions are not associated with the response to vaccination in RA patients treated with Kampo [36, 37].

In conclusion, we have demonstrated the changes in the titer of each anti-influenza antibody before and after vaccination in RA patients treated with Kampo formula. A low response to the vaccination was not observed compared with previous studies, and in the MTX-treated patients group, the response to vaccination was higher in our study than in previous reports. The present observations may open the way for further clinical trials to establish the efficacy for the influenza vaccination in RA patients treated with Kampo medicines.

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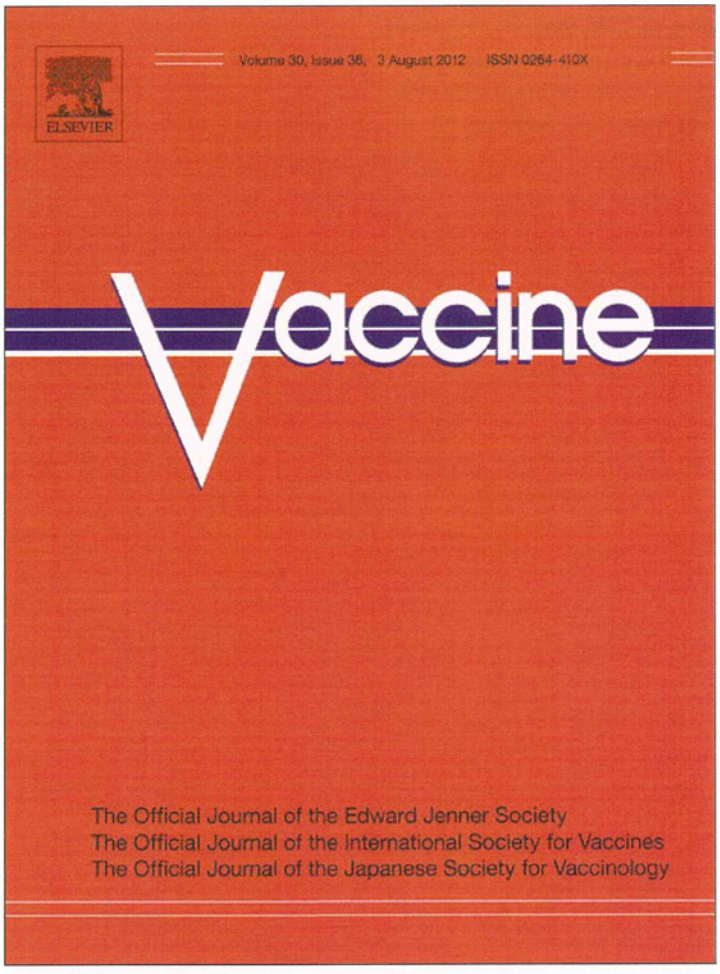

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Adjuvant effect of Japanese herbal medicines on the mucosal type 1 immune responses to human papillomavirus (HPV) E7 in mice immunized orally with *Lactobacillus*-based therapeutic HPV vaccine in a synergistic manner

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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 α4β7 [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 Heaithcare 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

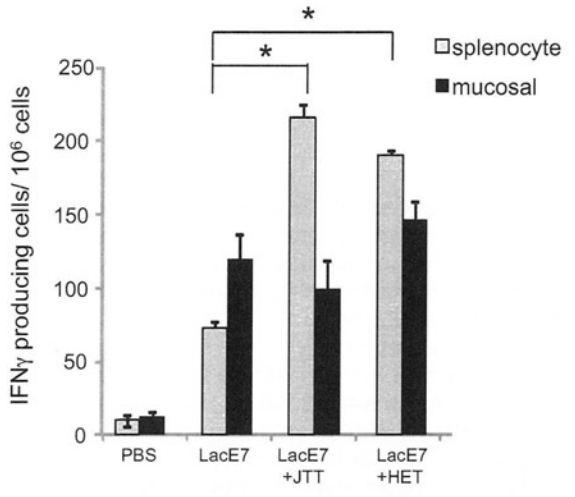

Fig. 1. Adjuvant effects of Japanese herbal medicines on type 1 T cell responses in mice orally immunized with Lac E7. The number of E7-specific IFNγ-producing cells among intestinal mucosal lymphocytes and splenocytes were assessed using ELISPOT assay. Five mice per group were immunized with LacE7 (1.0 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 ($p < 0.05$) ($n = 5$).

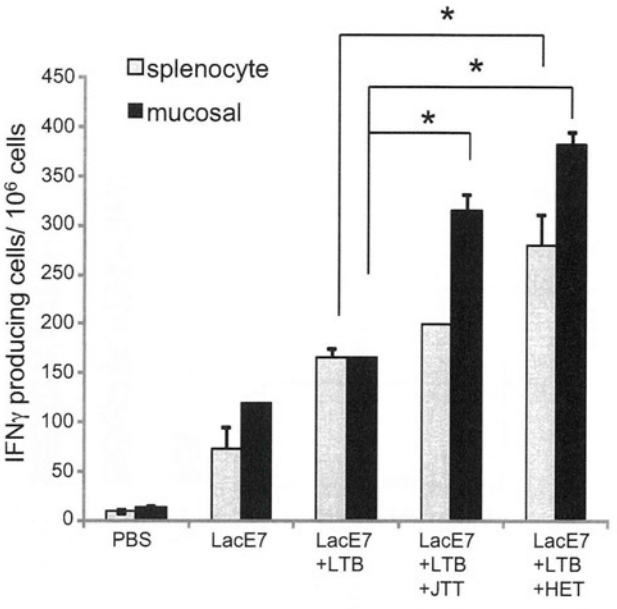

Fig. 2. Synergistic adjuvant effect of Japanese herbal medicines and LTB on type 1 T cell response. LTB (10 μg/head) was added to each LacE7 inoculum and administrated orally on the third day of each round of vaccination. This was performed in mice contemporaneously exposed to JTT, HET or control (no 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

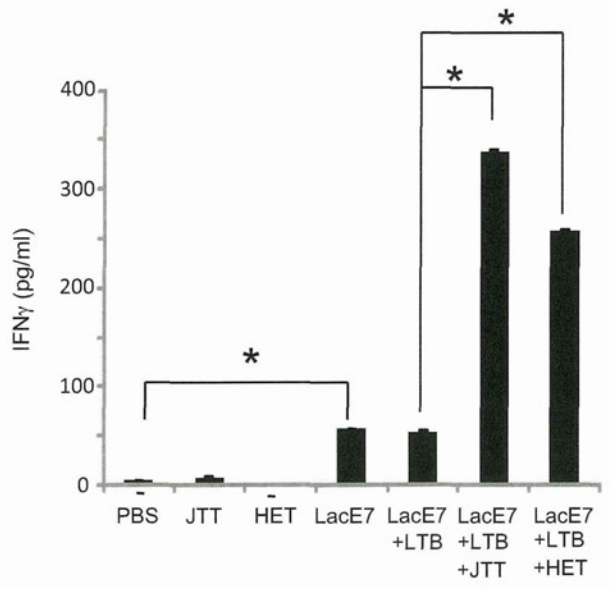

Fig. 3. IFNγ secretion into the intestinal compartment after immunization with LacE7 plus JTT or HET and LTB. IFNγ levels in the intestinal washes were measured by ELISA. The intestinal washes were collected at the same time points that were assessed in Fig. 1. Cytokine levels in each sample were normalized 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

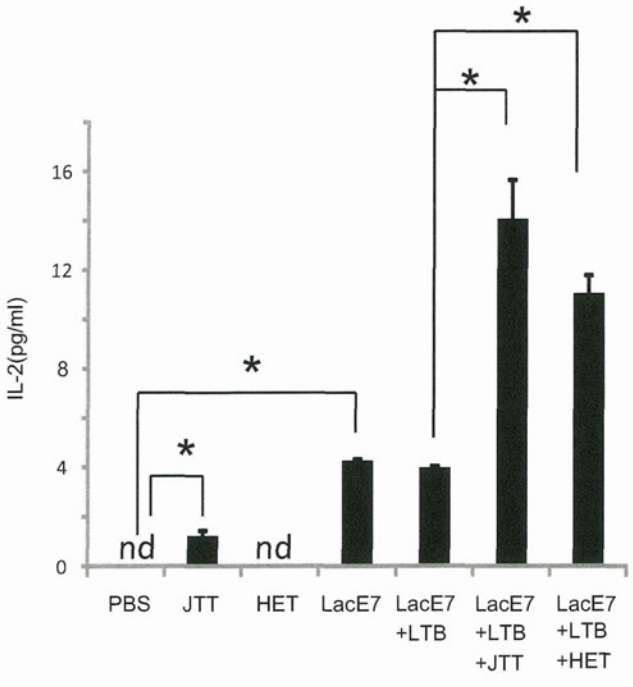

Fig. 4. IL-2 secretion into the intestinal compartment after immunization with LacE7 plus Japanese herbal medicine and LTB. IL-2 levels in the intestinal washes were measured by ELISA. The intestinal washes were collected at the same time points that were assessed in Fig. 1. Cytokine levels in each sample 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.

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Therapeutic Human Papillomavirus (HPV) Vaccines: A Novel Approach

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Abstract: Cervical cancer is the second largest cause of cancer-related death in women worldwide, and it occurs following persistent infection, sometimes for decades, with a specific subset of human papillomavirus (HPV) types; the approximately 13 oncogenic subtypes. Prophylactic vaccines against HPV infections hold promise for cost-effective reductions in the incidence of cervical cancer, but this may not be enough. Two prophylactic HPV vaccines are presently available and both contain L1 virus-like particles (VLPs) derived from the HPV subtypes most frequently associated with cervical cancer, HPV-16 and -18. Since the L1-VLP vaccines can only effectively prevent infection by the specific HPV subtype against which the vaccine was developed, cervical cancers caused by high-risk HPV subtypes other than HPV-16 and -18 may still occur in recipients of the current HPV vaccines. Furthermore, HPV vaccination coverage for adolescents is insufficient in most countries and therefore even HPV-16 and -18 infections are unlikely to be fully eradicated using the existing strategies. The development of HPV therapeutic vaccines remains essential. Many therapeutic vaccines aimed at clearing HPV-related cervical lesions have been developed and tested in patients with HPV16-positive cervical intraepithelial lesions (CIN) or cervical cancers. To date, definitive clinical efficacy and appropriate immunological responses have never been demonstrated for cervical neoplasia although promising results have been reported in patients with vulvar intraepithelial neoplasia. Here we discuss shortcomings of previous HPV therapeutic vaccine candidates and propose a novel vaccination strategy that leverages newly gained knowledge about mucosal immunity and the induction of mucosal immune responses.

Keywords: HPV therapeutic vaccine, mucosal vaccination, cervical mucosal immune system, E7-expressing lactobacillus-bases vaccine.

EPIDEMIOLOGY OF HPV INFECTION

At present, there are about 100 identified genotypes (types) of human papillomavirus (HPV) of which about 40 are genital HPV types that invade genital organs such as the uterine cervix, vaginal wall, vulva, and penis. Genital HPV types are classified into high-risk types commonly associated with cervical cancer and low-risk types known to cause condyloma acuminatum. This classification varies among researchers, but, in general, types 16/18/31/33/35/39/45/51/52/56/58/66/68 are classified as high-risk and 6/11/40/42/43/44/54/61/72 as low-risk [1]. Interestingly, the HPV type distribution varies depending on the stage of cervical neoplasia (Fig. **1**).

The HPV DNA detection rate in the genital organs of healthy adult females varies between advanced and developing countries but is approximately 20-40% collectively [2, 3]. In Japan, the HPV-positive rate in pregnant females aged 20-29 years has been reported to be 20-30%, which is similar to or higher than that among similarly aged females in the U.S [4]. The World Health Organization (WHO) has estimated an annual increase of 3 hundred million in the number of HPV carriers in the world [5, 6]. Overall HPV prevalence with normal cervical cytology was estimated to be 10.4 % [6]. Epidemiological data show HPV infection at least once during their lifespan in approximately 75 % of U.S. women [3]. Thus, HPV infection is common and can affect any female. Frequent sexual activity has been reported to increase the risk of HPV infection but this is not always the case [7].

NATURAL HISTORY OF CERVICAL INTRAEPITHELIAL NEOPLASIA

Natural history studies of CIN show that most infections and CIN lesions resolve spontaneously but some persist and progress to cervical cancer. The incidence of cervical intraepithelial neoplasia (corresponding to squamous intraepithelial lesion: SIL) is about 1 per 10 females with HPV infection [8]. The incidence of high grade SIL (corresponding to cervical intraepithelial neoplasia 2 and 3: CIN2-3) is about 3 per 10 females with low grade SIL, and that of CIN3 is about 1-2 per 10 females with low grade SIL [9]. Without treatment, the incidence of the progression of CIN3 to cervical cancer is about 30% [10]. Therefore, the incidence of the spontaneous development of cervical cancer is about 1 per 200-300 females with HPV infection. Factors associated with progression to cervical cancer in females with HPV infection have been extensively studied [1]. Many prospective studies have identified persistent HPV infection as the most important risk factor. They have also shown that persistent infection tends to occur in women with high risk HPV subtypes.

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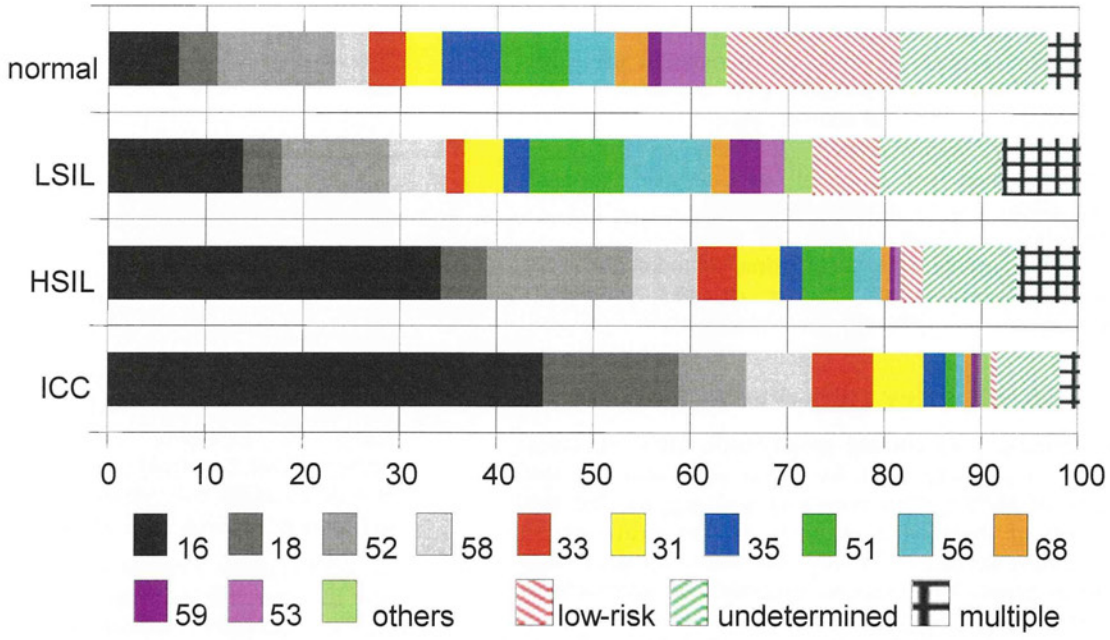

Fig. (1). HPV subtype distribution in cervical neoplastic lesions in Japan [18]. HPV16 and 18 are the most common subtypes found in invasive cervical cancer (ICC) but more than 40% of invasive lesions are associated with other oncogenic subtypes in Japan. HPV52 is the most common HPV subtype present among Japanese women with with normal cervical cytology [19].

Chronic virus proliferation induces the active proliferation/differentiation of infected epithelial cells, and some infected cells incidentally immortalize, which is the first step of carcinogenesis [1]. In contrast, transient infection involves short-term virus proliferation followed by the long-term latent presence of low copies of the viral genome in the basal cells of the genital epithelium [11]. Studies showing that HIV-infected women and patients who are under treatment with immunosuppressive agents have an increased incidence of CIN lesions [12, 13] suggest that cell-mediated immune response against HPV antigens is important in the control of HPV infection and progression to CIN. More controversial are the relative roles of systemic and local mucosal immune responses in HPV pathogenesis [14]. Trimble *et al.* reported that naturally occurring systemic immune responses to HPV antigens do not predict regression of CIN 2/3 lesions [15] but Nakagawa *et al.* demonstrated a positive association between systemic cell-mediated immune responses to HPV E6 and the regression of HPV/CIN [16].

SHORTCOMINGS OF THE CURRENT L1-VLP VACCINES

Theoretically, if HPV infection could be completely eradicated, HPV-associated cancers could be prevented. With this in mind, HPV vaccines began to be studied nearly 10 years ago. In 2002, Koutsky *et al.* were the first to show the clinical prophylactic effects of an HPV vaccine [17]. Soon thereafter, Merck in the United States and Glaxo Smith Kline (GSK) in Europe launched full-scale development of prophylactic vaccines against HPV. These products were approved and became commercially available just a few years ago. The vaccine antigens used by the two companies are virus-like particles (VLP) produced by overexpressing HPV16 L1 protein in yeast or insect cells. These particles have a 3-dimensional external structure similar to that of virus particles, but having no internal contents, they are not infective. The vaccine first reported by Koutsky *et al.* also used HPV16L1-VLP as an antigen.

One integral drawback of L1-VLP based vaccines is their negligible prophylactic effect on many HPV subtypes not specifically targeted by the vaccine [18]. For this reason, GSK and Merck developed cocktail vaccines composed of L1-VLPs corresponding to several HPV subtypes. The vaccine developed by Merck is a quadrivalent vaccine against HPV types 6, 11, 16, and 18 (Gardasil®) [19] and that developed by GSK is a bivalent vaccine against types 16 and 18 (Cervarix®) [20]. Unfortunately these L1-VLP vaccines are very specific and may not protect for long time against HPV types that exhibit very close genetic similarities to HPV-16 or -18, such as HPV-58 or -45 respectively. Ultimately, the most effective L1-VLP-based vaccines vaccines would be multivalent for the 13 described oncogenic HPV types. Such prophylactic vaccines would likely be much more expensive than their current counterparts.

HPV-16 or -18-related cervical cancers, which constitute less than 60% of all invasive cervical cancer cases in Japan [21], could be prevented if the appropriate subtype cocktail vaccine were available (Fig. **1**). However, the HPV subtype distribution in cervical cancer varies (60-70%) by worldwide location [22] and current vaccines are unable to address all oncogenic subtypes in even a single population. While current HPV vaccines are distributed without cost to the patient due to government subsidies or full coverage by insurance [23] these facile approaches will ultimately fail to eradicate the disease. Further, even with broad vaccination coverage, deficiencies in vaccine design mandate that even vaccinated females must continue cervical cancer screening.

The commercially available GSK and Merck HPV vaccines are indicated for uninfected females to prevent

HPV infection/spread. Due to the high prevalence of HPV infection, effective mass prophylactic vaccination strategies for uninfected females should include girls age 10 and above to predate the onset of sexual activity. Ph-III clinical studies in which females approximately 20 years of age were randomly inoculated with Gardasil® or Cervarix® revealed protective efficacy on the development of CIN2-3 associated with HPV-16 or -18 in 93-98% of vaccine-type naïve females who completed the vaccination protocol [24, 25]. However, intention-to-treat analysis revealed protective efficacy was only 19-30% for non-vaccine HPV subtypes [24, 25].

DEVELOPMENT OF HPV THERAPEUTIC VACCINES

The limitations of current prophylactic HPV vaccines demonstrate a pressing need for novel approaches to the eradication of HPV-related neoplasia and suggest that the development of therapeutic vaccines for the treatment of HPV-associated lesions will remain an important goal even if worldwide prophylactic vaccine programs are successfully implemented [26]. The past two decades has seen several inroads into the development of therapeutic HPV vaccines. The combined actions of the high-risk E6 and E7 oncoproteins are essential for the maintenance of the neoplastic phenotype and the evasion of apoptosis. Several functions have been described for E6 and E7. Initial observations revealed that E6 interacts with p53 and E7 interacts with Rb to block the activity of these tumour suppressors [1]. There are only two possible antigenic targets, E6 and E7, since these are the only viral proteins that will be expressed in all cancers and precursor lesions [1]. The approach of deliberate immunization with E6 and/or E7 of HPV 16 and 18 predominantly, and the generation of antigen-specific CTL as an immunotherapy for HPVassociated cancer has been tested with a wide array of potential vaccine delivery systems. Here we will summarize the results of the therapeutic vaccine clinical trials reported to (Table **1**) [14].

1. SGN-00101 (s.c.) is a fusion protein consisting of a heat shock protein (Hsp) from *Mycobacterium bovis* and HPV16 E7. The Ph-II study looking at the effects of SGN-00101 in women with CIN3 revealed histological regression to CIN1or less (complete remission: CR) in 13 (22.5%) of 58 cases, although immunological responses were not studied [27]. Another Ph-II study of the same agent administered to women with CIN showed the induction of cytotoxic T lymphocyte (CTL) against HPV16E7 in peripheral monocytes in 5 of 7 patients which obtained CR [28].
2. L1VLP-E7 (s.c.) is a vaccine using chimeric particles composed of HPV16 L1-VLP and E7. In the Ph-I/II study of women with CIN2-3, histological regression to CIN2 (partial remission; PR) was shown in 39% of vaccine recipients compared with 25 % of placebo recipients. This was not significant significant [29]. Clinical response was coupled with detectable cellular immune responses in some cases.
3. TA-HPV (i.m.) is a recombinant vaccinia virus expressing E6 and E7 of HPV-16 and -18. The Ph-II study of TA-HPV in women with vulvar intraepithelial neoplasia (VIN) revealed PR of lesions in 8 of 13 cases and responders also had an increase in lesion-infiltrating CD4 and CD8 positive cells [30].
4. TA-CIN (i.m.) is a fusion protein consisting of E6, E7 and L2 from HPV-16 and -18. The Ph-II study in women with VIN revealed CR or PR in only 6 of 29 cases. CTL against E6/E7 were induced in 4 of 29 cases [31]. Correlations between clinical efficacy and cellular immune responses to the vaccine remain unclear.
5. MVA-E2 (TGA4001) (intrauterine) is also a recombinant vaccinia virus expressing bovine papilloma virus (BPV) E2. A Ph-II study in subjects with CIN2-3 confirmed the down grade of CIN in some cases (19/34 cases) [32].
6. ZYC-101a (i.m.) is a DNA vaccine synthesized from proteins containing CTL epitopes against E6 and E7 of HPV-16 and -18. A Ph-III study was performed in subjects with CIN2-3. CR or PR was observed in 41% of vaccinated women and 27% of those receiving placebo. This was not a significant difference. Subset-analysis limited to those subjects aged 25 years or less revealed a statistically significant increase in the percentage of women with CR or PR in the vaccination group (72%) when compared to placebo controls (23%). However, no correlation was shown between CTL induction against E6/E7 and clinical effect [33].

Table 1. Clinical Trials of Therapeutic Vaccine for HPV-Associated Cervical Lesion

Trial Phase	Target Proteins	Vaccine Vectors	Inoculation	Target Types
Ph-I/II [27]	L1, E7	Chimera-VLP	S.C.	16
Ph-II [26]	E7	Hsp (SGN-00101]	S.C.	16
Ph-II [28]	E6, E7	Vaccinia virus (TA-HPV)	I.M.	16, 18
Ph-II [29]	L2, E6, E7	Fusion protein L2E6E7 (TA-CIN)	I.M.	16, 18
Ph-II [30]	BPV E2	Vaccinia virus (MVA-E2]	intrauteral	all
Ph-III [31]	E6, E7	plasmid vaccine (ZYC101a)	I.M.	16, 18
Ph-II [32]	E6, E7	Cocktailed Synthetic peptide	S.C.	16

S.C.: subcutaneous injection, I.M.: intramuscular injection, BPV: bovine papillomavirus.

7. Synthetic long-peptide vaccine (s.c.) is a peptide vaccine comprised of nine HPV16 E6 peptides and four HPV16 E7 peptides solubilized in incomplete Freund's adjuvant. A Ph-II study was performed in patients with VIN3. 5 of 20 patients demonstrated complete regression of their lesions [34].

In summary, no therapeutic HPV vaccines are presently available that exert significant clinical efficacy against CIN. Some of the tested therapeutic vaccines elicited systemic cellular immunity after intramuscular or subcutaneous injection, but none of the trials have assessed local cellular immune responses to vaccine antigen in the cervix.. The outcomes of vaccination strategies involving intramuscular or subcutaneous injection of E6/E7-based antigens for the treatment of VIN have been more promising [30, 31, 34]. We hypothesize that these findings are the direct result of the predicted poor response of cervical mucosal lesions to systemic cellular immune responses when compared to the effects of systemic immunity on epidermal lesions including those of VIN.

THE CERVICAL MUCOSAL IMMUNE SYSTEM AND HPV THERAPEUTIC VACCINES

Induction of adaptive cellular immune responses to HPV in the cervical mucosa is indispensable for treating cervical mucosal lesions such as CIN. Since precancerous lesion of the cervix develops essentially exclusively in the mucosal epithelium it would be predicted that intraepithelial lymphocytes (IELs) should be central to the elimination of CIN. To this poin, there are substantial differences between cellular and humoral immune responses in the female reproductive tract mucosa. It is well-known that intramuscular injection of L1-VLP based vaccines leads to systemic humoral immune responses characterized by the induction of anti-L1 IgG neutralizing antibody which leaks from the serum to protect the reproductive tract mucosa from HPV infection. However, the requirements for induction of mucosal cellular immune responses against microbial infected lesions differ from and are independent of those for systemic cellular immunity. Therefore, systemic intramuscular or subcutaneous vaccination strategies may be unsuitable for the induction of mucosal cellular immunity, at least in the reproductive tract mucosa.

In the uninduced state, the specific lymphocytes involved in mucosal immunity reside in the inductive sites of organized mucosa-associated lymphoid tissues (MALT); these are present in a variety of effector sites, including the mucosa of the intestine, respiratory tract and genital tract [35]. Efficient homing of lymphocytes to the gut is dependent on the homing receptors integrin $\alpha4\beta7$ and C-C chemokine receptor type 9 (CCR9). Lymphocyte-expressed integrin $\alpha4\beta7$ and CCR9 bind to their natural ligands, mucosal addressin cell adhesion molecule-1 (MAdCAM-1) and CCL25 (TECK), respectively, which are expressed on the cell surface of endothelial cells in submucosal post-capillary venules. In the intestine, mucosal dendritic cells (DCs) in gut-associated lymphoid tissues (GALT) regulate the expression of integrin $\alpha4\beta7$ on activated effector and regulatory lymphocytes in a retinoic acid-dependent manner [36]. Integrin $\alpha4\beta7$+ T cells reside the lamina propria in submucosa as lamina propria lymphocytes (LPL) and can differentiate into integrin $\alpha E\beta7$+ T cells upon exposure to TGF-β and expression of integrin $\alpha E\beta7$ facilitates retention of lymphocytes in the epithelium *via* interactions with E-cadherin [37] (Fig. **2**). Integrin $\alpha E\beta7$ is a specific marker of IELs residing in mucosal epithelia and those cells expressing this antigen on their surface were initially educated in the gut.

Several studies have demonstrated that human genital tract mucosa expresses MAdCAM-1 endogenously [38] and that GALT-derived integrin $\alpha4/E\beta7$+ T cells home to the genital mucosa [39-41]. This T cell homing and the expression of integrin αE increase in the presence of cervicitis and vaginitis [39, 40]. Although integrin β7+ mucosal T cells have been found in the cervical mucosa, a local inductive site (i.e., MALT) has never been demonstrated histologically [39, 40]. Taken together, GALT is thought to act as the inductive site for cervical IELs. GALT and the cervical mucosal connect through mucosa-specific T cells which express the homing receptors, integrin β7 and/or CCR9. Using flow cytometry, we have demonstrated that 25-30% of CD3-positive mucosal cervical lymphocytes are positive for the homing receptors integrin β7 and CCR9 and are thereby educated in GALT [41]. Approximately half of the integrin β7-positive T cells are CD45RO memory T cells while the other half are CD45RA effector T cells. Accumulation of integrin $\alpha E\beta7$+ IEL in CIN lesions varies markedly among patients and higher IEL numbers are associated with spontaneous regression of CIN [41]. These and related investigations have dramatically improved our understanding of cervical mucosal immunity which should hasten the development of a therapeutic HPV vaccine.

ORAL ADMINISTRATION OF HPV THERAPEUTIC VACCINES: A NOVEL APPROACH

Mucosal vaccination *via* oral administration of vaccine antigen is an effective method for the induction of mucosal immunity. Bermudez-Humaran *et al.* have evaluated the induction of CTL activity and the prevention/reduction of tumor formation following nasal or oral administration of live lactobacillus engineered to produce lactic acid-expressing HPV16E7 and IL-12, in tumor challenged murine models [42]. They found more marked induction of mucosal responses after nasal *vs* oral administration and a more effective induction of immunity when using *Lactobacillus plantarum vs Lactococcus lactis* [43]. Poo *et al.* have shown that oral immunization of C57BL/6 mice with *Lactobacillus casei* expressing HPV16 E7 reduces tumor formation induced by TC-1 cell administration. Immunization in these experiments elicited type 1 T cell immune responses to E7 in lymphocytes isolated from the spleen and from anogenital regional lymph nodes [44]. Although both studies used transmucosal immunization with Lactobacillus-based vaccines, they examined E7-specific systemic cellular immune response and regression of subcutaneous TC-1-induced tumors. These investigations provide no insight into mucosal cellular immune responses after immunization nor into the antigen specificity of mucosal lymphocytes. We have observed a marked induction of mucosal T cells possessing HPV16 E7-specific cellular immune recognition (E7-CMI) within intestinal mucosa after oral administration of *Lactobaccilus casei* expressing HPV16 E7 in mice [45].