The detection rate of HPV16 and 18 among women with cervical cancer in Japan is reported to be lower than that in other countries.[14] We used the latest age-dependent prevalence data, which consistently show that the younger population has a higher detection rate of HPV16 and 18 than the older population.[20] The prevention of cervical cancer in a young person shows larger QALYs gained than that of an older person because of the longer remaining life expectancy. Hence the effect of vaccine on cancer incidence or QALYs is not as low as might otherwise be expected.

The present study has several limitations. First, we assumed life-long lasting immunity acquired by the vaccine. The vaccine has only been recently introduced, and the latest evidence shows 7.3 years of efficacy and immunogenicity of the vaccine, which was derived from the population of the initial placebo-controlled study.[40] If additional vaccination is required to maintain immunity in the future, then programme costs are slightly underestimated. Second, there is no population-based survival data of women with cervical cancer by stages of FIGO. These data are essential when building a model. However, we managed to adopt and validate data from an existing Japanese regional cancer registry. Third, we did not incorporate the preferences of girls and their parents and the subsequent uptake of vaccine as a result of their preferences. Both effects and costs may be overestimated in that sense. Finally, we did not include the cost for campaigns to increase the coverage of screening and/or vaccination in this analysis, which may underestimate the programme costs but such a bias is minimal given the fact that the majority of costs is incurred by screening, vaccination and treatment interventions.

Vaccination for HPV is attracting considerable policy attention now as a strategy for cervical cancer prevention in Japan. Our analysis showed that increasing the rate of the current screening strategy would halve cancer incidence with a similar cost to the current screening strategy, though vaccination strategies may also be cost effective. We suggest further efforts to expand the current screening programme regardless of what support is provided for vaccination.

Some of the reasons why Pap smear coverage is so low in Japan relate to a lack of knowledge and from the fact that the financial support of the screening programme from the Ministry of Health, Labour and Welfare was discontinued because it was included in the general ones in 1998. Most cities, towns and villages decided to reduce the cost for the screening programme.[41–43] Free tickets for the Pap smear were provided under supplemental budgets for 2009. Distributing free tickets to a target population of certain ages showed a significant increase in the coverage rate by 2.8 times.[44] We need to continue endeavours to increase coverage by effective interventions such as providing free tickets and undertaking awareness campaigns. The involvement of gynaecologists in school education will also support the enhancement of knowledge about cervical cancer prevention and help to increase the coverage rate of screening as has been seen in other countries.[45,46]

Our analysis showed that introducing the HPV vaccination for all 11-year-old girls would reduce cervical cancer incidence to 33.9% with a net cost of only 49,000 yen per person (taking into account the social burden of cancer). Vaccinating all 11-year-old girls would cost 33.7 billion yen. Our analysis showed the cost-effectiveness of vaccination and that it would save future costs. It is important to give priority to policy which is evidence based medically and economically. If the prevalence of HPV infection is reduced as a result of universal vaccination, as our model predicts, then it may be possible to extend the interval between routine screens or to increase the age at which screening is first offered, as suggested in other cost-effectiveness studies.[34,47] The use of the HPV-DNA test in the screening programme is one choice that should be evaluated in the future.

In conclusion, the introduction of HPV vaccine in Japan is cost-effective as in other countries. It is more cost-effective to increase the coverage of the Pap smear along with the universal administration of HPV vaccine. Only by doing so, can the scarce healthcare resources be efficiently and effectively used to reduce the burden from cervical cancer in Japan.

Disclosure of interests

None of the authors have any conflicts of interest to declare.

Contribution to authorship

NY contributed to the study design of the current paper, model construction, data acquisition, data analysis and interpretation, drafting and revising the manuscript. RM contributed to the study design of the current paper, model construction, results interpretation and revising the manuscript. PJ contributed to the model construction, results interpretation and the critical review of the manuscript. YO contributed to the study design of the current paper. KK contributed to the model construction, data acquisition and interpretation of the results. KS and YT contributed to the study design of the current paper and interpretation of the results. All authors approved the final version of the manuscript.

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CASE REPORT

Interstitial pneumonitis induced by pegylated liposomal doxorubicin in a patient with recurrent ovarian cancer

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Abstract Interstitial pneumonitis after treatment with pegylated liposomal doxorubicin (PLD) has been rarely reported. We describe herein a case of interstitial pneumonitis in a 49-year-old woman with relapsed ovarian carcinoma treated with PLD. Twenty-five days after the second administration of PLD, she presented with fever and dry cough, and chest CT scans revealed bilateral interstitial infiltrates and ground-glass opacities. She was diagnosed to have interstitial pneumonitis induced by PLD. Steroid therapy improved her symptoms.

Keywords Interstitial pneumonitis · Pegylated liposomal doxorubicin · Drug induced · Japanese · Ovarian cancer

Introduction

Pegylated liposomal doxorubicin (PLD) is an active drug in recurrent ovarian cancer as demonstrated in trials in the second-line chemotherapy [1–3]. It has been designed to enhance the efficacy and to reduce the toxicities of doxorubicin such as cardiotoxicity, hematologic toxicity, and alopecia by using a unique delivery system: a polyethylene glycol coat [4, 5]. Whereas hand-foot syndrome and planter palmar erythema are widely recognized as adverse effects of PLD, few cases of interstitial pneumonitis after treatment with PLD have been reported. Here, we describe a case of interstitial pneumonitis induced by PLD.

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Case report

A 48-year-old woman (gravida 4, para 3) with recurrent ovarian cancer was started on third-line chemotherapy with PLD (50 mg/m^2/4 weeks). She was initially diagnosed in February 2009 and underwent complete debulking surgery for a stage IIIC serous ovarian adenocarcinoma. Postoperatively, she received adjuvant chemotherapy with six cycles of paclitaxel (175 mg/m^2) and carboplatin (AUC 6). Four months later, because of peritoneum dissemination and elevation of CA125, she was treated with weekly CPT-11 (95 mg/m^2/week) with progressive disease after four cycles. In April 2010, PLD was given under her excellent performance status.

Twenty-three days after the first administration of PLD, she developed a fever from which she recovered without any treatment. However, 25 days after the second administration of PLD, she presented to our hospital with fever, chill, dry cough, and dyspnea (grade 3 according to Common terminology criteria for adverse events, version 4.0). Physical examination was remarkable for bilateral fine crackles at the lung bases. A chest X-ray and chest CT scans revealed bilateral interstitial infiltrates and ground-glass opacities, though chest CT scans performed before PLD therapy showed clear lung field (Fig. 1a, b). Oxygen saturation by pulse oximetry was 89% on room air and arterial blood gas analysis showed hypoxia (FiO_2 0.32, PaO_2 90.5 mmHg, alveolar-arterial oxygen gradient 94.9 mmHg). Laboratory analysis revealed white blood cells of 2,500/μl with 78% neutrophils, lactate dehydrogenase of 347 IU/l, C-reactive protein of 14.32 mg/dl, and Krebs von den Lungen-6 (KL-6) of 227 U/ml.

Her clinical course and laboratory data indicated that she has interstitial pneumonitis probably induced by PLD. She had not received granulocyte colony-stimulating

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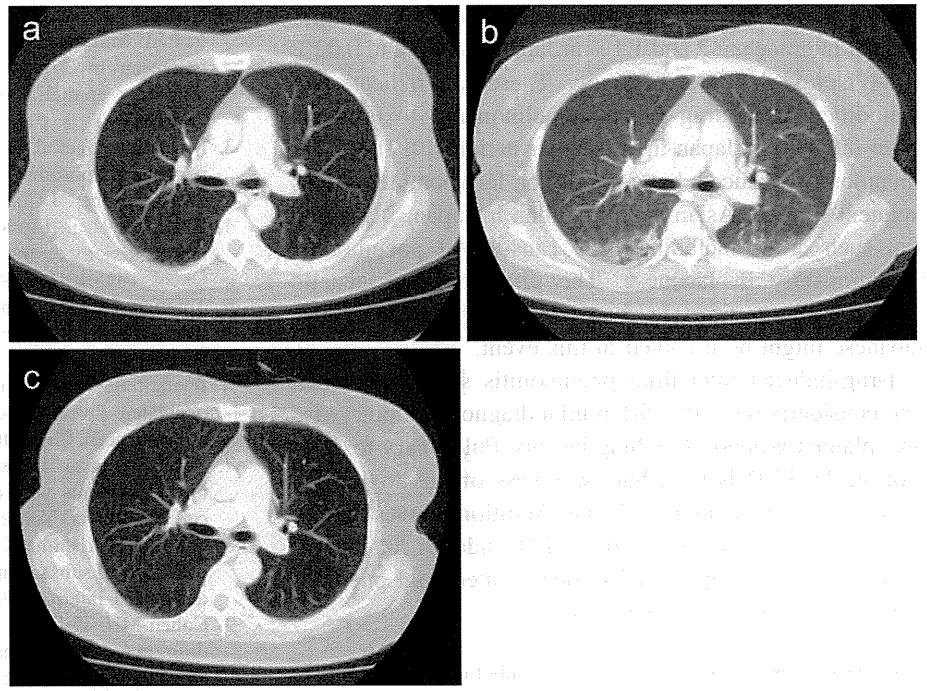

Fig. 1 **a** Chest computed tomography (CT) scan before PLD therapy showed clear lung field. **b** Twenty-six days after second administration of PLD, CT revealed bilateral interstitial infiltrates and ground-glass opacities. **c** Two months after steroid therapy, CT showed significant improvement

factor, which could induce interstitial pneumonitis. In addition to PLD, she received ascorbic acid, pyridoxal phosphate hydrate, rebamipide, and brotizolam. As they were all unlikely to induce interstitial pneumonitis, administration of these drugs except PLD was continued. The patient was treated with intravenous methylprednisolone 500 mg/day for 3 days. Azithromycin 1,000 mg per os and intravenous cefepime 4 g/day were administrated until all examinations of infection proved to be negative, including blood culture, β-D-glucan, influenza antigen detection, urinary pneumococcal antigen test, Chlamydia IgA/IgG, candida antibody assays, and galactomannan antigen of aspergillosis.

After the steroid pulse therapy, symptoms resolved promptly and lung function tests improved remarkably. Two months after the diagnosis of interstitial pneumonitis, a chest CT scan showed significant improvement (Fig. 1c). PLD was discontinued and her chemotherapy regimen was changed to docetaxel (70 mg/m^2). She has not shown any respiratory symptoms after cessation of PLD. Currently, she is alive with disease 24 months after the surgery and undergoing fifth-line chemotherapy.

Discussion

Pegylated liposomal doxorubicin is a reformulated version of doxorubicin, which takes the active agent doxorubicin and places it into a phospholipid bilayer called a liposome and another outer layer of methoxypolyethylene glycol. This coating allows PLD to evade detection and destruction by the immune system and to remain longer in the blood circulation.

PLD has a different toxicity profile compared with free doxorubicin. Though cumulative cardiac toxicities are unique to free doxorubicin, cardiac toxicities associated with PLD are rarely reported. Toxicities relatively unique to PLD are hand-foot syndrome or plantar palmar erythema (PPE), which are rarely reported with free doxorubicin.

It is reported that lung toxicity induced by doxorubicin is rare. Several cases of interstitial pneumonitis associated with doxorubicin or PLD have been described [6, 7]. It was unclear whether the lung toxicities were directly attributable to doxorubicin in published case reports, because all patients were concurrently receiving other agents, mostly antineoplastic drugs, which were also implicated in causing lung toxicity.

In our case, though the symptoms were initially severe, discontinuation of PLD and steroid therapy immediately resolved them. Serum KL-6 levels have been reported to correlate with grade of interstitial lung disease [8]. Normal serum KL-6 level in this case might associate with her excellent clinical course.

Two possible mechanisms of drug-induced interstitial pneumonitis have been described, one of which is the direct toxicity of the drug to the pulmonary organ and the other is immunological mechanism, although the etiology of PLD-induced interstitial pneumonitis is unclear.

Drug-induced pulmonary toxicity in Japan got a great deal of attention because of pulmonary toxicity induced by molecular-targeted chemotherapeutic drugs, gefitinib and an antirheumatic drug, leflunomide. It is reported that the

rates of interstitial lung disease associated with gefitinib and leflunomide are 2 and 1.1% in Japan and 0.3 and 0.02% in the United States, respectively. These data indicate that chemotherapeutic-drug-induced pulmonary toxicity is more frequent in Japan than in other nations [9, 10]. Fatal pneumonitis induced by gefitinib or leflunomide is less frequent in other Asian countries than in Japan. It may be that such drugs including PLD cause fatal pneumonitis predominantly in Japanese. The differences of genetic background or lifestyle between Japanese and non-Japanese might be involved in this event.

Drug-induced interstitial pneumonitis should be taken into consideration in the differential diagnosis of otherwise unexplained ground-glass lung lesions. Pulmonary toxicity induced by PLD is rare, but awareness of this toxicity is important, since it could be lethal. Additional investigation is required to elucidate how PLD induces interstitial pneumonitis or whether PLD-induced interstitial pneumonitis is more frequent in Japanese.

Conflict of interest No author has any conflict of interest.

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LETTER TO THE EDITOR

Second-line chemotherapy with docetaxel and carboplatin in paclitaxel and platinum-pretreated ovarian, fallopian tube, and peritoneal cancer

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Abstract We retrospectively evaluated the efficacy and toxicity of docetaxel and carboplatin in patients with platinum and paclitaxel-pretreated recurrent ovarian, fallopian tube, and peritoneal cancer. Forty-two women (38 with ovarian cancer, 1 with fallopian tube cancer, 3 with peritoneal cancer) whose cancer had progressed within 12 months of their last treatment with both a platinum agent and paclitaxel were treated with docetaxel (70 mg/m^2, day 1) and carboplatin (area under the curve of 4–6, day 1). Thirty-four patients had measurable disease. The objective response rate was 23% within 0–6 months of the progression-free interval, 50% within 6–12 months, and 32% (11 of 34 patients) for both groups. The median time to tumor progression was 28, 49, 34 weeks, and the median overall survival time was 94, 224, 111 weeks, respectively. The most common toxicity was grade 3/4 neutropenia (98% of patients), with 15 episodes (8.4% of courses) of neutropenic fever. The main nonhematologic toxicity was hypersensitivity; 7 patients (17%) required discontinuation of the therapy. The results of our study indicate that the combination of docetaxel and carboplatin is effective against recurrent ovarian, fallopian tube, and peritoneal cancer with progression-free interval of 6–12 months from previous treatment by paclitaxel and platinum. On the other hand, single-agent chemotherapy would be better than this regimen considering its low response rate and severe hematological toxicity for patients with progression-free interval less than 6 months.

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Keywords Docetaxel · Carboplatin · Chemotherapy · Early progression · Recurrent ovarian cancer

The standard regimen as second-line chemotherapy in recurrent ovarian cancer has not been established, especially in the patients with a short progression-free interval from the previous treatment. Docetaxel is an active drug as second-line chemotherapy for recurrent ovarian cancer as well as pegylated liposomal doxorubicin, irinotecan, topotecan, gemcitabine, and etoposide [1].

The purpose of this study was to evaluate activity and toxicity of the combination of docetaxel and carboplatin retrospectively in patients with paclitaxel and platinum resistant (progression-free interval less than 6 months) and partially resistant (progression-free interval of 6–12 months) ovarian, fallopian tube, and peritoneal cancers. Forty-two women (38 with ovarian cancer, 1 with fallopian tube cancer, 3 with peritoneal cancer) whose cancer had progressed within 12 months of their last treatment with both a platinum agent and paclitaxel were treated with docetaxel (70 mg/m^2, day 1) and carboplatin (area under the curve of 4–6, day 1). Thirty-four (81%) patients had measurable disease. Twenty-six (62%) patients had experienced progression of disease within less than 6 months of their last treatment, whereas 16 patients (38%) within 6–12 months. The median number of courses of treatment per patient was 4.5 (range: 1–8 courses). The median follow-up period was 107 weeks (range: 9–373 weeks). The objective response rate was 23% within 0–6 months of the progression-free interval, 50% within 6–12 months, and 32% (11 of 34 patients) for both groups. The median time to tumor progression was 28, 49, and 34 weeks, and the median overall survival time was 94, 224, and 111 weeks, respectively. The most common toxicity was grade 3/4 neutropenia (98% of patients), with 15 episodes

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(8.4% of courses) of neutropenic fever. The main nonhematologic toxicity was hypersensitivity; 7 patients (17%) required discontinuation of the therapy. On the other hand, grade 2/3 neuropathy was observed only in two (4.8%) patients.

Several chemotherapeutic agents such as pegylated liposomal doxorubicin, topotecan, irinotecan, gemcitabine, and etoposide have been used in the treatment of platinum-resistant disease with response rates in the range 10–15% [2–5]. The results from our study about overall response rate are in line with other chemotherapeutic agents. Notably, our data about median time to tumor progression and overall survival are longer than the previously reported data of other regimens.

The results of our study indicate that the combination of docetaxel and carboplatin is effective against recurrent ovarian, fallopian tube, and peritoneal cancer with progression-free interval of 6–12 months from previous treatment by paclitaxel and platinum. On the other hand, single-agent chemotherapy would be better than this regimen considering its low response rate and severe hematological toxicity for patients with progression-free interval less than 6 months. However, chemotherapy with docetaxel and carboplatin may improve time to tumor progression and overall survival time in these cases; this regimen can be an alternative in patients whose hematological toxicity is relatively weak at their previous treatment.

Conflict of interest None.

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Characterization of Gut-Derived Intraepithelial Lymphocyte (IEL) Residing in Human Papillomavirus (HPV)-Infected Intraepithelial Neoplastic Lesions

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Problem
Mucosal T cells are the most likely direct effectors in host anti-human papillomavirus adaptive immunity and regression of cervical intraepithelial neoplasia (CIN) lesions. There are no studies addressing intraepithelial lymphocytes (IELs) in CIN lesions.

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

Results
A median of 74% of cervical lymphocytes were $CD3^+$ T cells. Populations of integrin $\alpha E\beta 7^+$ IEL in CIN lesions varied markedly among patients (6–57%). Approximately half of integrin $\beta 7^+$ T cells were CD45RA-negative memory T cells. The number of integrin $\alpha E\beta 7^+$ cells among cervical T cells was significantly higher in CIN regressors when compared to non-regressors.

Conclusion
Higher cervical IEL numbers are associated with spontaneous regression of CIN. Accumulation of cervical integrin $\alpha E\beta 7^+$ IEL may be necessary for local adaptive effector functions.

Introduction

Lymphocytes involved in the mucosal immune system are found in the inductive sites of organized mucosa-associated lymphoid tissues (MALT) and in a variety of effector sites such as the mucosa of the intestine, respiratory tract, and genital tract.[1] The efficient homing of lymphocytes to the gut is dependent on the homing receptors integrin α4β7 and C-C chemokine receptor type 9 (CCR9). Lymphocyte-expressed integrin α4β7 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.[1] 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.[1–3] Mucosal T cells expressing integrin $\alpha4\beta7^+$ are known to circulate in peripheral blood from inductive sites and to home to the lamina propria (LP) at effector sites via $\alpha4\beta7$-MAdCAM-1 and CCR9-CCL25 interactions.[4] Integrin $\alpha4\beta7^+$ T cells can differentiate into $\alpha E\beta7^+$ T cells upon exposure to TGF-β,[5] and the expression of integrin $\alpha E\beta7$ facilitates the retention of lymphocytes in the epithelium via interactions with E-cadherin.[4] Integrin $\alpha E\beta7$ is a specific marker of intraepithelial lymphocytes (IELs) residing in mucosal epithelia, and those cells expressing this antigen on their surface were initially educated in the gut.

The cervical mucosa is a very common site for pathogen invasion and is the primary transmission site for human papillomavirus (HPV), *Chlamydia trachomatis*, and human immune deficiency virus type 1 (HIV-1). A well-organized mucosal defense system in the cervical mucosa is critical to human health. Mucosal epithelial cells in the human cervix are active participants in such immunological protection.[6] However, the lymphocytes populating the cervical mucosal tissues, especially cervical IELs, have been poorly studied. Mucosal T cells in the murine genital tract express a large amount of integrin $\alpha4\beta7$ on their cell surface,[7] and MAdCAM-1 is expressed on endothelial cells in the submucosa of murine fallopian tubes infected with *C. trachomatis*.[8] Several studies have demonstrated that human genital mucosa expresses MAdCAM-1 endogenously[9] and that GALT-derived integrin $\alpha4/E\beta7^+$ T cells home to the genital mucosa.[10,11] This T-cell homing and the expression of integrin αE increase in the presence of cervicitis and vaginitis.[10,11] Although integrin $\beta7^+$ mucosal T cells have been found in the cervical mucosa, a local inductive site (i.e., MALT) has never been demonstrated histologically.[11] We hypothesized that GALT may act as the inductive site for cervical IELs.

Human papillomavirus infection is a major cause of cervical cancer, and its precursor lesion, cervical intraepithelial neoplasia (CIN), develops in the epithelium. Natural history studies of CIN[12,13] show that most infections and CIN lesions resolve spontaneously but some persist 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[14,15] 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 the HPV pathogenesis. Trimble et al.[16] reported that naturally occurring systemic immune responses to HPV antigens do not predict regression of CIN 2/3 lesions, but Nakagawa et al.[17] demonstrated a positive association between systemic cell-mediated immune responses to HPV E6 and HPV/CIN regression.

We studied the local mucosal cell–mediated immune response to HPV antigens by characterizing cervical mucosal immune cells collected non-invasively, using only a cytobrush. We confirmed that the collected $CD3^+$ cervical T cells were intraepithelial in origin (integrin $\alpha E\beta7^+$ IELs). Approximately half of the integrin $\beta7^+$ T cells were memory T cells. Finally, integrin $\beta7^+$ intraepithelial T cells increased significantly in the patients whose CIN lesions regressed spontaneously regardless of HPV genotype.

Materials and methods

Study Population

Cervical cell samples were collected using a cytobrush from 86 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. Samples for HPV genotyping were collected at the first follow-up examination after diagnosis. Cervical lymphocytes were collected from non-menstruating patients at their latest follow-up visit. To study the potential association between cervical IEL characteristics and CIN progression, CIN patients with the 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 to 4-month intervals. Thirteen patients were enrolled in the regression group, and the median

follow-up duration was 27 (12–38) months. Thirteen 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 24 (12–40.5) months.

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.[18] 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 86 patients in our study was shown in Table I. Here, 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 International Agency for Research on Cancer multicenter study.[19]

Table I Human Papillomavirus (HPV) Genotype Distribution

HPV type	Total numbers (%)
16	19 (18.4)
18	7 (6.8)
31	2 (1.9)
33	1 (1.0)
35	1 (1.0)
39	1 (1.0)
45	1 (1.0)
51	7 (6.8)
52	20 (19.4)
53	4 (3.9)
56	3 (2.9)
58	12 (11.7)
59	3 (2.9)
68	3 (2.9)
82	1 (1.0)
6	2 (1.9)
54	1 (1.0)
55	1 (1.0)
66	4 (3.9)
69	1 (1.0)
70	3 (2.9)
83	3 (2.9)
84	2 (1.9)
Total	103 (100)

Patients infected with multiple HPV types were included. Of 86 patients, 32 (37%) 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.[19]

Collection and Processing of Cervical Specimens

Cervical cells were collected using a Digene cytobrush as described previously.[20] The cytobrush was inserted into the cervical os and rotated several times. The cytobrush was placed in a 15-mL tube containing R10 media [RPMI-1640 medium, supplemented with 10% fetal calf serum (FCS), 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 centrifuged at 330 × *g* for 4 min. The pellet was resuspended in 10 mL of 40% Percoll, 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 >95%, as confirmed by trypan blue exclusion test, and fresh samples were immediately used for further analysis.

Immunolabeling and Flow Cytometry

Cervical immune cell preparations were immunolabeled, incubated on ice for 30 min, washed twice with FACS buffer (10% FCS, 1 mM EDTA, and 10 mM NaN_3) and fixed by adding paraformaldehyde in PBS to a final concentration of 1%.

The following fluorochrome-conjugated mouse monoclonal antibodies specific for human leukocyte surface antigens were used: a fluorescein isothiocyanate (FITC)-conjugated pan leukocyte marker (FITC-anti CD45), a B lymphocyte marker (FITC-anti CD19), a cytotoxic T-cell marker (FITC-anti CD8), a helper T-cell marker (FITC-anti CD4), an integrin β7 marker (FITC-anti integrin β7), a phycoerythrin (PE)-conjugated integrin α4 marker (PE-anti integrin α4), an integrin αE marker (PE-anti integrin αE), a C-C chemokine receptor type 9 marker (PE-anti CCR9), a marker for naïve cells (PE-anti CD45RA), a phycoerythrin cyanine 5 (PC5)-conjugated pan T lymphocyte marker (PC5-anti CD3), a natural killer cell marker (PC5-anti CD56), and an allophycocyanin (APC)-conjugated pan T lymphocyte marker (APC-anti CD3). Cell preparations were labeled in parallel with appropriate isotype control

antibodies. Antibodies were purchased from eBioscience (San Diego, CA, USA) and Beckman Coulter (Brea, CA, USA). Data were acquired using three-color flow cytometry on FACSCalibar (Becton-Dickinson, Texarkana, TX, USA). The positions of lymphocytes and monocytes were determined on the forward scatter versus side scatter (SSC) profile. The positions of pan-lymphocytes and T lymphocytes were determined by CD45 and CD3 gating, respectively. As the percentage of B cells among cervical lymphocytes is known to be low (less than a few percentage) when compared to the 20% level seen in peripheral blood,[20] the presence of elevated $CD19^+$ B cells in cervical specimens would indicate contamination with peripheral blood. For our investigations, cervical samples with more than 3% B cells were excluded from analysis.

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 test or Fisher's exact test was applied for matched paired comparisons. *P*-values ≤ 0.05 were considered significant.

Results

Purification of Cervical Leukocytes Collected from CIN Lesions

To characterize mucosal cellular immune responses in HPV-infected lesions, cervical samples, including exfoliated epithelial cells and cervical lymphocytes, were collected from CIN lesions positive for any HPV genotype using a cytobrush. Cervical samples were fractionated over a discontinuous Percoll density gradient to remove cervical epithelial cells, and the layer between Percoll and culture medium was collected. Cervical lymphocytes were identified among isolated cells using standard SSC and CD45 gating (Fig. 1). Approximately 10^4–10^5 $CD45^+$ cells were isolated from patients' cervices. $CD45^+$ cells primarily consisted of lymphocytes (Fig. 1, circle) and granulocytes (Fig. 1, square). A minority of the cells included in the square in Fig. 1 were monocytes (data not shown). Two representative cases are provided in Fig. 1: the upper panel represents a patient with numerous granulocytes and a relatively small population of $CD45^+$ lymphocytes (10%), whereas the lower panel represents a patient with few granulocytes and a high number of lymphocytes (30%).

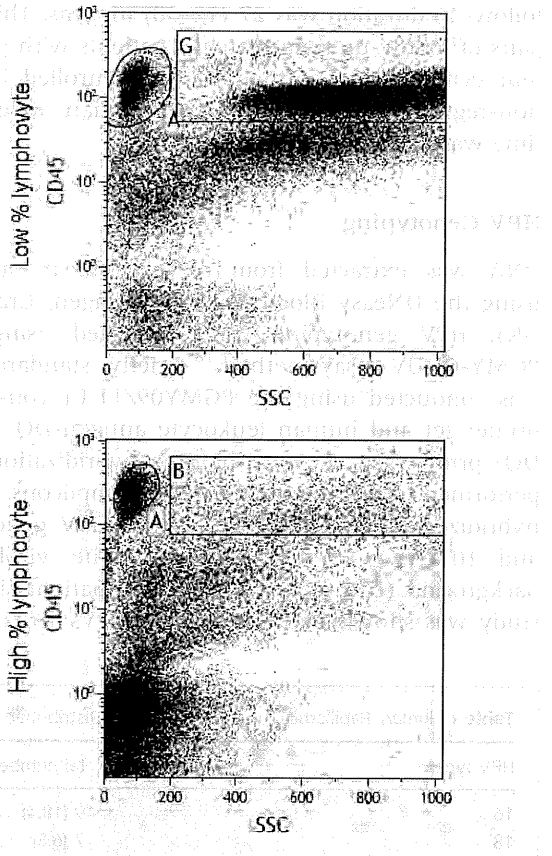

Fig. 1 Flow cytometric analysis of cervical mucosal cells using CD45/SSC gating. Processed cervical specimens were analyzed by flow cytometry and CD45/SSC gating. $CD45^+$ cervical leukocytes are comprised of lymphocytes (circle) and granulocytes/monocytes (square). Upper and lower panels were representative of patients with low (about 10%) and high (about 30%) numbers of lymphocytes among their $CD45^+$ cervical leukocytes, respectively. The absolute number of isolated cervical lymphocytes remained relatively constant among study subjects.

Characterization of Cervical T Cells in CIN Lesions

The majority of cervical lymphocytes isolated from CIN lesions were $CD3^+$ T cells [median 74% (IQR: 59–82)]. $CD19^+$ B cells were rarely found [median 0.45% (IQR: 0.04–1.40)]. In Fig. 2, CD3-gated cervical T cells were characterized by flow cytometry, and each median, IQR, and maximum/minimum range is indicated using horizontal lines, boxes, and vertical length lines, respectively. A median of 54%

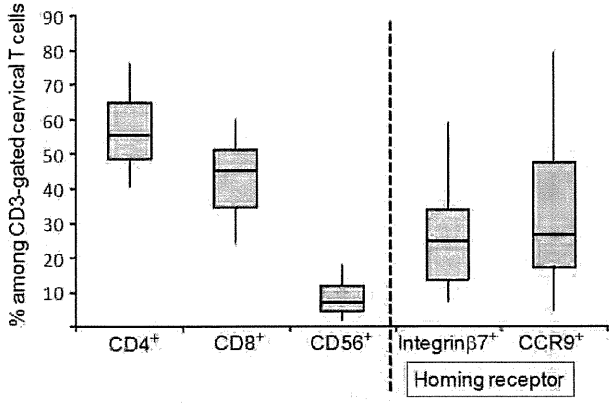

Fig. 2 Characterization of the cervical $CD3^+$ lymphocytes. CD3-gated cervical T cells consisted of $CD3^+$ $CD4^+$ T cells [median 54% (IQR: 49–65), $n = 28$], $CD3^+$ $CD8^+$ T cells [median 46% (IQR: 35–51), $n = 28$], and $CD3^+$ $CD56^+$ natural killer T cells [median 5.6% (IQR: 4.5–12), $n = 17$]. Twenty-four percentage (IQR: 13–34, $n = 43$) and 27% (IQR: 17–47, $n = 27$) of cervical T cells were integrin $\beta7^+$ and $CCR9^+$, respectively. Each median, IQR, and maximum/minimum range is indicated using horizontal lines, boxes, and vertical length lines, respectively.

(IQR: 49–65) of cervical T cells were $CD3^+$ and $CD4^+$, while a median of 46% (IQR: 35–51) expressed CD3 and CD8, demonstrating that $CD8^+$ T cells are relatively abundant among cervical T cells. The CD4/CD8 ratio of 1.15 in the cervix was clearly lower than the value of 2.0 found in peripheral blood. Among $CD3^+$ cells, a median of 5.6% (IQR: 4.5–12) were $CD56^+$ natural killer T (NKT) cells.

Those cervical $CD3^+$ T cells that were originally derived in the gut were defined by expression of the gut mucosa–specific cell-surface antigens integrin $\beta7^+$ and $CCR9^+$. A median of 24% (IQR: 13–34) of cervical T cells expressed integrin β7 and 27% (IQR: 17–47) expressed CCR9 (Fig. 2).

Notably, more than 90% (median: 99.1, IQR: 95.3–100) of the integrin $\beta7^+$ cells co-expressed the αE subunit (integrin $\alpha E\beta7^+$ cells; Fig. 3a). Integrin $\alpha4^+$ cells were rarely present among the integrin $\beta7^+$ cells (Fig. 3b). Approximately 40% (median: 40.1, IQR: 33.2–44.2) of cervical integrin $\beta7^+$ cells were integrin $\alpha E\beta7^+$ $CCR9^+$ double positive (Fig. 3c).

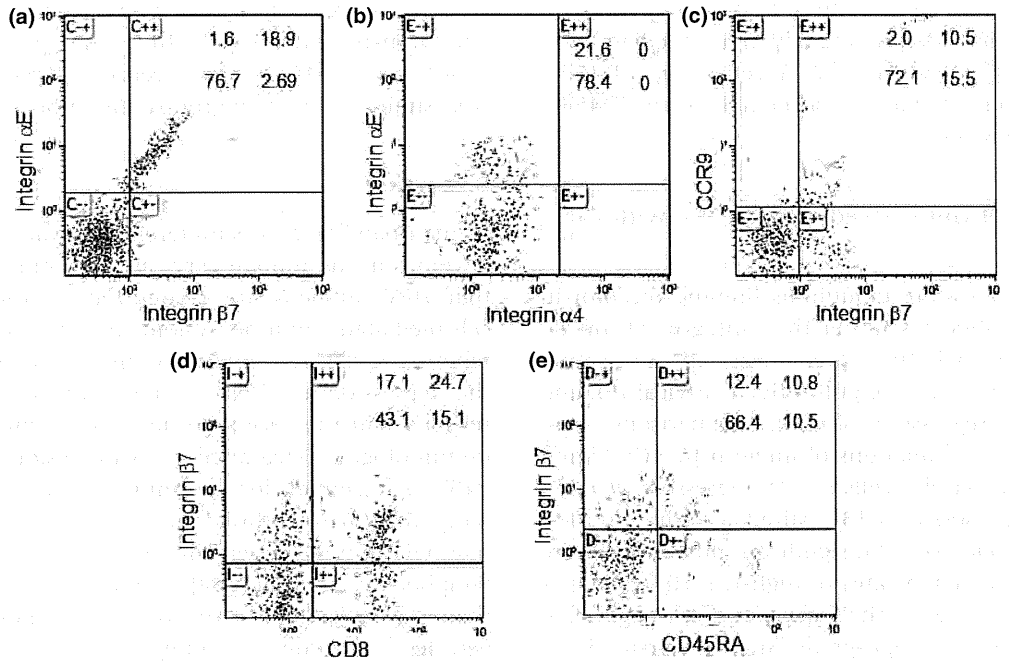

Fig. 3 Characterization of CD8, CD45RA, and homing receptors specific for gut-derived mucosal T cells among $CD3^+$ cervical T cells. Representative flow cytometry analyses of CD3-gated cervical T cells. (a) More than 90% of integrin $\beta7^+$ cervical T cells were integrin αE^+ intraepithelial lymphocyte. (b) Integrin $\alpha4^+$ LPL were negligible in our cervical samples. (c) Among integrin $\beta7^+$ cells, approximately 40% were $CCR9^+$. (d) Forty-two percentage of total cervical T cells and 53% of integrin $\beta7^+$ T cells were $CD8^+$. (e) About half of the integrin $\beta7^+$ T cells were CD45RA-negative memory cells.

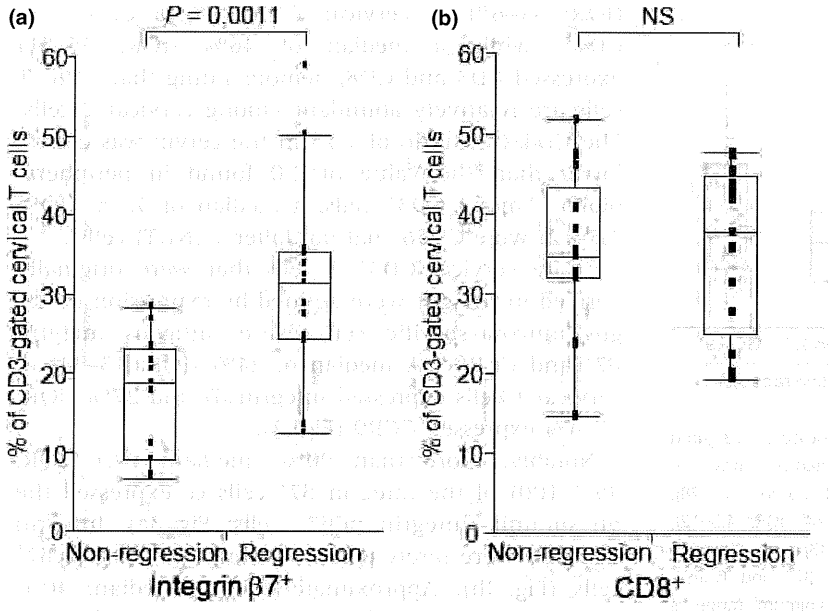

Fig. 4 Association between gut-derived cervical intraepithelial lymphocyte and cervical intraepithelial neoplasia (CIN) regression. Populations of integrin $\beta7^+$ (a) and $CD8^+$ (b) cells among $CD3^+$ cervical T cells were compared between CIN regression (n = 13) and non-regression (n = 13) groups, paired according to follow-up duration. A P-value ≤0.05 was considered significant using Wilcoxon rank test comparisons.

$CD8^+$ memory T cells are essential for adaptive cytotoxic immune responses to CIN.[21,22] Among our patients with CIN, the proportion of integrin $\beta7^+$ cervical T cells that expressed CD8 [median 53% (IQR: 28–69)] was greater than that for total cervical T cells (Fig. 3d). Approximately half [median 43% (IQR: 31–57)] of integrin $\beta7^+$ T cells were CD45RO memory T cells, while the other half were CD45RA effector T cells (Fig. 3e).

Association of Gut-derived Cervical IEL with CIN Course

Integrin β7 is a more ubiquitous homing receptor in mucosal lymphocytes rather than integrin αE or α4. To determine whether there was an association between the presence of gut-derived cervical IEL and spontaneous regression of CIN, comparisons were made between populations of integrin $\beta7^+$ $CD3^+$ and $CD8^+$ $CD3^+$ cervical T cells in CIN regressors (n = 13) and non-regressors (n = 13), paired according to their duration of follow-up. No significant differences were seen in the detection rates of high-risk HPV (69 versus 77%, P = 0.50), the squamous intraepithelial lesion (SIL) grade (high-grade SIL: 54 versus 54%, P = 0.65), and the median ages (34 years old versus 35) of patients in the regression and non-regression groups. The percentage of integrin $\beta7^+$ cervical T cells varied from 6 to 57% among the 26 study subjects. Among regressors, integrin $\beta7^+$ cervical T cells comprised a median of 31.6% (IQR: 24.5–35.5) of $CD3^+$ cervical T cells; the rate among non-regressors was 18.8% (IQR: 9.2–23.3), P = 0.0011 (Fig. 4a). In contrast, there was no difference in populations of $CD8^+$ $CD3^+$ cervical T cells between CIN regressors and non-regressors (Fig. 4b). The proportion of $CCR9^+$ and $CD45RA^+$ $CD3^+$ cervical T cells was likewise similar in the two groups (data not shown).

Discussion

Human papillomavirus preferentially infects, and CIN develops in the human cervical epithelium. It is clear that HPV antigens are recognized by the systemic cell-mediated immune system, but remains unclear whether systemic cellular immune responses predict the regression of CIN.[16,17] Local mucosal immune responses in the cervix are likely to be important in immunological clearance of CIN lesions. Integrin $\alpha4\beta7$ is essential for recruiting activated mucosal lymphocytes from the circulation into local LP in a manner entirely dependent on interaction between lymphocyte integrin $\alpha4\beta7$ and the MAdCAM-1 that is constitutively expressed on LP post-capillary venules.[23] In contrast, integrin αE (CD103) β7 is expressed by only 2% of circulating blood lymphocytes, but more than 90% of IEL and a minority of lamina propria lymphocyte (LPL); its ligand is E-cadherin expressed on the epithelial cells.[24] Activated integrin $\alpha4\beta7^+$ T cells differentiate within the

LP into integrin $\alpha E\beta 7^+$ T cells upon exposure to TGF-β locally secreted by epithelial cells.[5] Binding of integrin $\alpha E\beta 7$ to E-cadherin provokes retention of the activated IEL within the epithelium. Recognition of target epithelial cells by IELs is important in the initiation of cytolytic effector function by activated IELs and modulation of adaptive immune responses to control potentially destructive epithelial immunity. Adhesion of integrin $\alpha E\beta 7^+$ IEL to epithelial E-cadherin is promoted by CCL25–CCR9 interactions.[4] This suggests that, when compared to integrin $\alpha 4\beta 7^+$ LPL, integrin $\alpha E\beta 7^+$ IELs may be more directly linked to essential adaptive immune responses to target epithelial cells at local effector sites.

Several studies have reported that integrin $\alpha 4\beta 7$ is expressed on gut-derived mucosal lymphocytes within the cervix.[9,11] However, our data indicate that more than 90% of integrin $\beta 7^+$ T cells were positive for integrin αE and few express α4. Pudney et al.[10] have shown using immunohistochemistry that integrin $\alpha E\beta 7^+$ lymphocytes are primarily located in the epithelium of the ectocervix and often occur as focal accumulations in the LP of the transformation zone. Our brushing methodology enables us to preferentially collect cervical mucosal lymphocytes from the epithelium and occasionally from the LP. Others who have recently reported that nearly all cervical tissue T cells are integrin $\alpha 4\beta 7^{+9}$ used cervical tissue specimens and equally valuable methodologies that would be expected to isolate cells from deeper within the cervical tissue, possibly favoring isolation of LPL over cells tightly adhered to the epithelium.

Our cervical samples were contaminated by numerous granulocytes, a finding supported by several previous studies using cervical mucosa unlike peripheral blood samples.[10,11] Granulocyte contamination variability was likely the result of differing levels of cervical inflammation among patients. Although the number of lymphocytes among $CD45^+$ cervical leukocytes varied from 10 to 30%, the absolute number of cervical lymphocytes present in a sample appeared to be relatively constant and independent of patient source. The efficient homing of lymphocytes to the gut is dependent on the homing receptors integrin β7 and CCR9. We showed that integrin β7 and CCR9 did not always co-express. This agrees with reports showing that expression of the mucosal homing receptors, integrin β7 and CCR9, is not always linked, but instead depends on lymphocyte differentiation and the location of the effector sites infiltrated by these cells.[25,26]

Expression of MAdCAM-1 is essential for trafficking of integrin $\alpha 4\beta 7^+$ lymphocytes into the LP, while the expression of E-cadherin on the epithelium is essential for the retention of integrin $\alpha E\beta 7^+$ lymphocytes. Inflammation of the mucosa enhances MAdCAM-1 expression on the endothelial cells of post-capillary venules in the genital tract,[8] and inflammatory changes are often observed in CIN when compared with normal cervical mucosa.[27,28] Trimble et al.[9] reported that MAdCAM-1 expression correlates with non-specific $CD8^+$ LPL infiltration of the LP and CIN regression. In our sampled IELs, there was no association between $CD8^+$ cells and CIN regression. Studies have also demonstrated that oncoproteins from high-risk HPV subtypes downregulate E-cadherin expression in CIN lesions and that this downregulation is closely associated with disease progression.[29–31] E-cadherin plays an important role in the maintenance of normal adhesion in epithelial sites and its loss is associated with poor prognosis for many tumors other than CIN.[32] The downregulation of E-cadherin may interfere with the retention of integrin $\alpha E\beta 7^+$ T cells in CIN lesions, and our results suggest that IEL retention varies among patients with CIN. We have shown that populations of integrin $\alpha E\beta 7^+$ IEL in CIN lesions vary markedly among patients and that higher IEL numbers are associated with spontaneous regression of CIN. Although HPV-specific cytotoxic T lymphocyte activity was not investigated here, the accumulation of integrin $\alpha E\beta 7^+$ IEL in CIN lesions and their association with CIN regression suggests these cells, rather than non-specific $CD8^+$ T cells, may have important local effector functions in the cervical epithelium. In the present study, the adaptive immune system was focused, but the innate immune responses play equally important roles in controlling HPV infection. Daud et al.[33] has recently reported the mechanism of interference with innate immune system by HPV16, dampened toll-like receptor expression, which results in the viral persistence. The interaction of innate with adaptive immunity at the local mucosa should be investigated.

In summary, our report is the first to specifically phenotype cervical IEL in CIN lesions. Our results indicate that the presence of elevated numbers of gut-derived integrin $\alpha E\beta 7^+$ IELs in specimens gathered from patients with CIN using a cervical cytobrush may represent a possible biomarker for CIN regression. Sampling of cervical IEL using this methodology is relatively non-invasive and techni-

cally easier than the isolation of cervical LPL from tissue biopsies. Future investigations using our sampling methods will focus on HPV-specific cell-mediated immune responses by cervical IELs isolated from patients with CIN. These and related investigations should improve our understanding of cervical mucosal immunity and hasten the development of a therapeutic HPV vaccine.

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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