Table 1 Characteristics of the study subjects

	Cytology and histology		P values[†]
	Biopsy-negative LSIL ($n = 64$)[a]	LSIL/CIN1 ($n = 479$)	
Age (years)			
Mean (SD)	38.8 (9.2)	36.2 (7.7)	
18–29	11 (17.2%)	95 (19.8%)	0.07
30–39	21 (32.8%)	215 (44.9%)	
40+	32 (50.0%)	169 (35.3%)	
HPV typing			
Positive for high-risk types[b]	36 (62.1%)	359 (78.0)	0.01
Negative for high-risk types	22 (37.9%)	101 (22.0%)	
Positive for any HPV	48 (77.4%)	405 (88.0%)	0.02
Negative for any HPV	14 (22.6%)	55 (12.0%)	
Smoking			
Never smokers	37 (63.8%)	222 (51.3%)	0.07
Smokers	21 (36.2%)	211 (48.7%)	
Current smokers	16 (27.6%)	143 (33.0%)	
Former smokers	5 (8.6%)	68 (15.7%)	
Number of lifetime sexual partners			
1	23 (39.6%)	79 (18.1%)	0.001
2–3	13 (22.4%)	129 (29.5%)	
4+	22 (37.9%)	229 (52.4%)	
Age at first sexual intercourse (years)			
≤20	12 (20.3%)	147 (34.2%)	0.06
21–23	26 (44.1%)	179 (41.6%)	
≥24	21 (35.6%)	104 (24.2%)	
IgG antibodies to *Chlamydia trachomatis*			
Low	27 (45.0%)	166 (36.1%)	0.25
Mid	20 (33.3%)	150 (32.6%)	
High	13 (21.7%)	144 (31.3%)	
IgG antibodies to HSV2			
Low	23 (38.3%)	158 (34.3%)	0.82
Mid	19 (31.6%)	150 (32.6%)	
High	18 (30.0%)	152 (33.0%)	

[†] These P value were calculated by the χ^2 test

[a] Biopsy-negative LSIL denotes women with LSILs that had a negative biopsy result at the initial colposcopy

[b] HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68 were classified into high-risk HPV types

biopsies were taken at the initial colposcopy and there was no difference in the number of biopsies between the two groups.

Patients were monitored by cytologic and colposcopic testing at intervals of 3–4 months. Among women with biopsy-negative LSILs, no case was diagnosed with CIN3+ within 2 years; the difference in the cumulative risk of CIN3+ diagnosed within the next 2 years between the two groups was marginally significant (0 vs. 5.5%; $P = 0.07$ by log-rank test; Fig. a). In women with biopsy-negative LSILs, the majority of cytologic regression occurred within 12 months. The cumulative probability of cytologic regressions within 12 months was much higher in women with biopsy-negative LSILs than in women with LSIL/CIN1 (71.2 vs. 48.6%; $P = 0.0001$; Fig. b). The 2-year rate of cytologic regression was also significantly different between the two groups (75.1 vs. 64.0%; $P = 0.003$). Cytologic regression occurred more quickly in women with biopsy-negative LSILs than in women with LSIL/CIN1 (median time to regression: 6.3 vs. 12.4 months). In the women with biopsy-negative LSILs, the 12-month cumulative probability of cytologic regression was similar between hrHPV-positive and -negative women (67.3 vs. 74.4%; $P = 0.74$); median time to regression was also similar between hrHPV-positive and -negative women (5.4 vs. 7.7 months; $P = 0.45$; Fig. a). In women with LSIL/CIN1, however, detection of hrHPVs significantly influenced the 12-month rate of cytologic regression (hrHPV-positive [45.2%] vs. hrHPV-negative [62.6%]; $P = 0.006$; Fig. b).

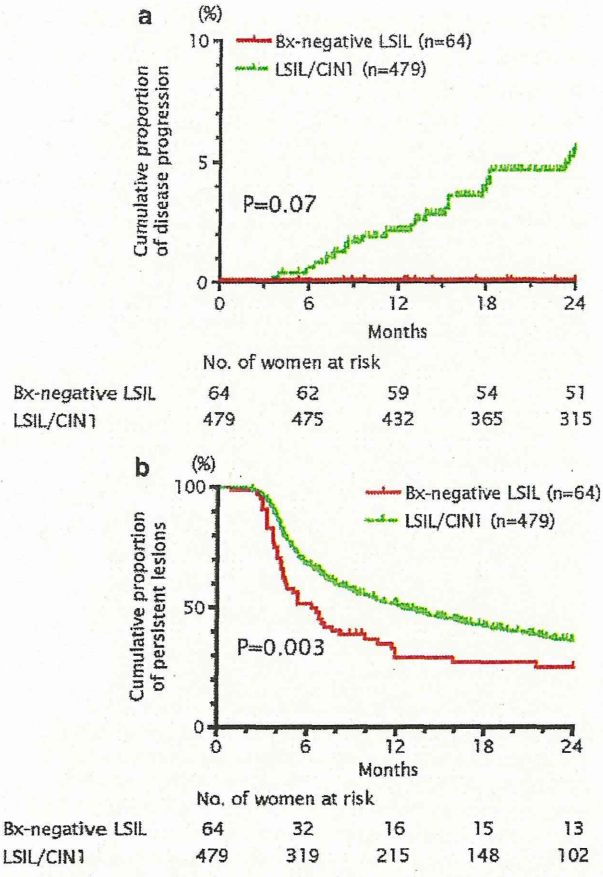

Fig. 1 Cumulative probabilities of CIN3+ diagnosis and cytologic regression within 2 years. A Kaplan–Meier plot was used to estimate the cumulative probabilities of CIN3+ diagnosis (**a**) and cytologic regression (**b**) within 2 years among women with biopsy-negative LSILs (*solid line*) or LSIL/CIN1 (*dashed line*). *P* values were calculated by the log-rank test

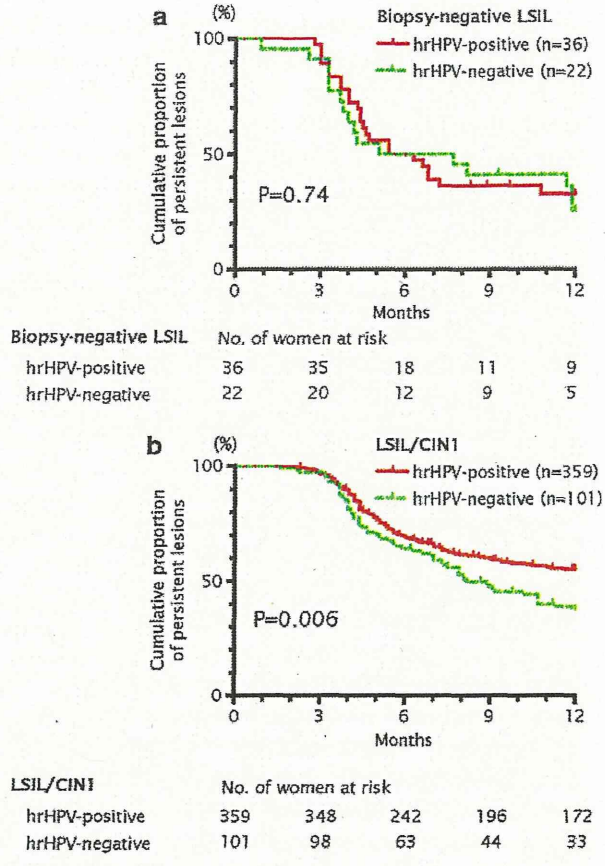

Fig. 2 Cumulative probabilities of cytologic regression within 12 months according to detection of hrHPVs. A Kaplan–Meier plot was used to estimate the cumulative probabilities of cytologic regression within 12 months among women with biopsy-negative LSILs (**a**) or LSIL/CIN1 (**b**) according to hrHPV detection. *P* values were calculated by the log-rank test

Discussion

Colposcopy-directed biopsies are recommended for women with LSIL cytology, primarily to exclude a high-grade lesion. Although approximately 15–30% of those women have a negative biopsy result [2, 3], they are routinely subjected to follow-up because of uncertainty about the risk of precancerous lesions missed by a colposcopic biopsy. In the present study, women with a biopsy-negative LSIL (i.e., "unconfirmed" LSIL) were at substantially low risk of CIN3 or cancer diagnosed within the following 2 years. The women with biopsy-negative LSILs were also significantly more likely to have cytologic regression than women with LSILs underlying CIN1. Some cases of biopsy-negative LSIL may be based on false-positive cytology because the percentage of women negative for any HPV was significantly higher in the biopsy-negative LSIL group than in the LSIL/CIN1 group. Additionally or alternatively, biopsy-negative LSILs may represent currently regressing lesions. This may be supported partially by the higher percentages of women in the biopsy-negative LSIL group who did not have cervical cancer risk factors, such as detection of hrHPVs, smoking, higher sexual activity and infections with *Chlamydia trachomatis* [13–16]. Several studies have reported that LSIL is more likely to regress to normal cytology among hrHPV-negative women or women who never smoked [5, 6, 17]. Interestingly, the 12-month regression rate of biopsy-negative LSIL was high, even among hrHPV-positive women. Low-grade lesions currently regressing to normal cytology may be difficult to confirm by colposcopy-guided biopsies because of the small lesion size, lower-grade colposcopic impression and/or weak pathologic findings.

Data on the natural course of biopsy-negative LSILs are limited. Pretorius et al. [18] reported that the subsequent risk of CIN3+ among women with histologically unconfirmed atypical squamous cells of undetermined significance (ASC-US) or LSIL cytology was low (1.8%). This

result was consistent with our observation; however, it was based on retrospective analyses of previous data including ASC-US cytology. In the ALTS (ASCUS-LSIL Triage Study) report [2], the risk of CIN3+ diagnosed within 2 years after unconfirmed LSIL was higher compared with the present study (6.1 vs. 0%). The difference between our results and the ALTS data may be explained by the difference in study design between the two studies. In the ALTS study, all women had an exit colposcopy and biopsy at 2 years after the semiannual follow-up by repeated cytology. Although our study subjects received both cytologic and colposcopic examinations at each visit at 3- to 4-month intervals, we did not routinely perform a colposcopic biopsy 2 years later. This may have resulted in an underestimation of the 2-year risk for CIN3+ in our study. Additionally, the sensitivity of the enrollment colposcopy may have affected the results from these two prospective studies. Recent studies have showed that initial colposcopy-directed biopsy are not as sensitive as we had previously assumed [19]. Thus, at least two directed biopsies, random biopsy or endocervical curettage are recommend to increase the sensitivity of the initial colposcopy [20–22]. In the ALTS study, 77.6% of women had null or only one biopsy at enrollment colposcopy [20]. By contrast, two (or more) biopsies were taken at entry in our study subjects. The number of biopsies may have increased the risk of misclassification errors of cervical lesions at enrollment. Although central pathologic review systems were employed in both studies, the limitation of histopathologic diagnosis (i.e., poor reproducibility in CIN grading) may also have affected disease classification at enrollment and during follow-up [7, 8, 23].

The current US guidelines advise that women with LSIL cytology and a histologic diagnosis of CIN1 or less should be followed with repeated cytology at 6 and 12 months or, alternatively, hrHPV testing at 12 months [4]. Our data also confirmed that these management strategies are sufficiently safe. A previous study reported that there was no significant difference in the subsequent risk of CIN2/3 between women with no disease documented by initial colposcopy-directed biopsy and women with histologically confirmed CIN1 [24]. However, the study was based on retrospective analyses, which was limited by the small sample size (negative biopsy $n = 43$; CIN1 $n = 30$) and included women with various cytologic abnormality profiles. In the present study, the risk of CIN3+ diagnosed within the following 2 years and the likelihood of LSIL regression were obviously different between women with biopsy-negative LSILs and women with LSIL/CIN1. The 2-year follow-up in ALTS of women with CIN1 or less has indicated that the subsequent risk of CIN2 or higher varies little with respect to the findings at the initial colposcopy [2]. However, when the analysis was confined to the risk of CIN3 or higher among women with LSILs, there was a marginal tendency for a higher risk of subsequent CIN3 that was associated with CIN1 compared with <CIN1 (10.5 vs. 6.1%). Based on these observations, the follow-up strategy for women with biopsy-negative LSILs may be better differentiated from that for women with LSIL/CIN1 results in terms of quality-of-life and cost. Our data suggest that follow-up by repeated cytology at 12 months may be appropriate for women with biopsy-negative LSIL when two or more colposcopy-directed biopsies are taken at the initial colposcopy.

In conclusion, the risk of CIN3+ diagnosed within 2 years was low in women with biopsy-negative LSILs; furthermore, approximately 70% showed cytologic regression within 12 months, regardless of HPV testing results. Our data suggest that biopsy-negative LSILs may represent false-positive cytology or currently regressing lesions rather than lesions missed by colposcopy. However, the sample size of the present study was small; thus, to confirm our results, further prospective studies with larger sample sizes will be needed.

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Conflict of interest We declare that we have no conflict of interest relevant to this article. The supporting organization played no role in the design and implementation of the study; the collection, management, analysis, and interpretation of the data; and the preparation, review, or approval of the manuscript.

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Do Neutralizing Antibody Responses Generated by Human Papillomavirus Infections Favor a Better Outcome of Low-Grade Cervical Lesions?

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To determine the role of neutralizing antibody generated by human papillomavirus (HPV) infections, baseline levels of serum neutralizing antibodies directed against HPV 16 and cervical HPV DNA were determined in 242 unvaccinated women with low-grade cervical abnormalities, who were then monitored by cytology and colposcopy every 4 months. In women infected with HPV 16 (n = 42), abnormal cytology persisted longer in those positive for HPV 16-specific neutralizing antibodies at baseline (median time to cytological regression: 23.8 vs. 7.2 months). Progression to cervical precancer (cervical intraepithelial neoplasia grade 3) within 5 years occurred only among women carrying HPV 16-specific neutralizing antibodies ($P = 0.03$, log-rank test). In women infected with types other than HPV 16 (n = 200), detection of HPV 16-specific neutralizing antibodies was not correlated with disease outcome. In conclusion, development of specific neutralizing antibodies following natural HPV 16 infection did not favor a better outcome of low-grade cervical lesions induced by HPV 16 or by other types; rather, detection of neutralizing antibodies generated by current infection may reflect viral persistence and thus help identify those who are at high risk of disease progression. ***J. Med. Virol. 84:1128–1134, 2012.***

KEY WORDS: human papillomavirus; neutralizing antibody; low-grade squamous intraepithelial lesion; progression; cervical intraepithelial neoplasia

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INTRODUCTION

Human papillomavirus type 16 (HPV 16) is the most common genotype detected in cervical cancer worldwide [Muñoz et al., 2003]. HPV 16 virus-like particles (VLPs) are obtained through the self-assembly of the major capsid protein L1 expressed in insect or yeast cells. HPV 16 VLP-based ELISA has been used to detect HPV 16 capsid antibody responses following natural infections [Frazer, 2009]. More than half of women infected by HPV 16 produce serum IgG antibodies [Carter et al., 2000]. HPV 16 VLP IgG antibodies are more commonly detected in women who have been persistently positive for HPV 16 DNA at different time points [de Gruijl et al., 1997; Sasagawa et al., 1998]. In women not infected currently with HPV 16, the presence of ELISA antibody responses to HPV 16 VLPs correlated with a significantly reduced risk of subsequent new HPV 16 infection, although the risk was reduced by only 50% compared to that of seronegative women [Ho et al., 2002; Safaeian et al., 2010].

In the case of many viruses, the presence of serum neutralizing antibodies is a correlate of immune protection. Recently, production of HPV 16 pseudovirions encapsidating a secreted alkaline phosphate (SEAP) reporter gene has allowed efficient measurement of specific HPV 16 neutralizing antibodies in women infected with HPV 16 or vaccinated with HPV 16 VLPs. Although serum antibody titer measured by VLP-based ELISA correlated with the neutralizing antibody titer, the neutralization assay was found to be more sensitive and type-specific than the VLP-based ELISA detecting total antibodies to HPV 16 capsids [Pastrana et al., 2004]. Immunization with VLP-based vaccines elicits neutralizing antibodies and protects against new infections in animals and humans [Stanley, 2010]. However, there have been few studies assessing the role of neutralizing antibodies in women infected currently with HPV or in those with cervical diseases. The neutralization activity against HPV may prevent persistence of infection by inhibiting viral spread between cervical epithelial cells or act as markers of host immunity responses to control cervical HPV infection.

Most low-grade cervical lesions are known to regress spontaneously, whereas only a small fraction progress to precancer lesions and cervical cancer [Östör, 1993; Melnikow et al., 1998; Holowaty et al., 1999]. In the current study, baseline serum neutralizing antibodies against HPV 16 were measured to assess possible association with clinical outcomes of low-grade cervical lesions induced by HPV 16 or other HPV types.

MATERIALS AND METHODS

Study Design

Follow-up data from the Japan HPV And Cervical Cancer (JHACC) study (a prospective nonintervention cohort study conducted to assess regression and progression of low-grade cervical abnormalities [Matsumoto et al., 2010, 2011]) were used in this study. Among a total of 570 study subjects, 242 women, whose serum samples were available for the HPV 16 neutralizing assay, were enrolled in the present study. Details of the design and methods have been provided elsewhere [Matsumoto et al., 2011]. Briefly, women with low-grade squamous intraepithelial lesions which were diagnosed as cervical intraepithelial neoplasia grade 2 or less on initial biopsy were recruited from nine hospitals. All volunteer patients provided written informed consent. Cervical smears were classified according to the Bethesda System [Solomon et al., 2001]. On enrollment, two cervical punch biopsy specimens were collected and stained with hematoxylin and eosin (H&E). Histological diagnosis was based on the World Health Organization (WHO) classification system. All cytological and histological specimens were reviewed by two cytopathologists (Y. H. and Masafumi Tsuzuku) and two pathologists (R. F. and T. K.). Levels of serum neutralizing antibodies to HPV 16 and cervical HPV DNA were also determined on enrollment. Researchers conducting the assays were blinded to the corresponding clinical data collected from the patients. The patients meeting enrollment criteria were followed at 3- to 4-month intervals with cytology and colposcopic examination. Cervical HPV DNA was determined at 24 months after enrollment. To avoid interference with the natural course of the disease, a cervical biopsy was performed only when the follow-up findings suggested progression to cervical intraepithelial neoplasia grade 3 or worse. The two cytopathologists and two pathologists reviewed all the cytological and histological specimens collected for diagnosis of disease progression. Progression was defined histologically as the presence of cervical intraepithelial neoplasia grade 3 lesions. In this analysis, regression was defined as normal colposcopy and at least two consecutive negative cervical smears. Lesions which did not regress or progress during the follow-up period were defined as persistent lesions.

The study protocol was approved by the ethics and research review boards of the participating institutions.

HPV Genotyping

HPV DNA in cervical samples was determined by the polymerase chain reaction (PCR), as described previously [Yoshikawa et al., 1991]. Briefly, exfoliated ecto- and endocervical cells were placed in a tube containing 1 ml phosphate buffered saline and stored at −30°C until DNA extraction. Total cellular DNA was extracted by a standard sodium dodecyl sulfate (SDS)–proteinase K procedure. HPV DNA was amplified by PCR using consensus-primers (L1C1/L1C2 + L1C2M) for the HPV L1 region. A reaction mixture without template DNA was included in every

set of PCR runs as a negative control. In addition, primers for a fragment of the β-actin gene were used as an internal control to assess the quality and quantity of template DNA in each PCR specimen. HPV types were identified by restriction fragment length polymorphism (RFLP), which has been shown to distinguish at least 26 types of genital HPVs [Nagano et al., 1996].

To minimize misclassification errors of HPV 16 DNA, HPV 16 infection was confirmed by PCR-amplified DNA sequencing using HPV 16 E6-specific synthetic primers (5-GACATTTTATGCACCAAAAG-3 and 5-GTATCT CCATGCATGATTAC-3, spanning nt 75–575) [Matsumoto et al., 2000].

Preparation of Pseudovirions

Dr. J. T. Schiller kindly donated three plasmids for this study: pYSEAP, expressing secreted alkaline phosphatase (SEAP); p16L1h, expressing HPV 16 L1; and p16L2h, expressing HPV 16 L2. 293FT cells (Invitrogen, Carlsbad, CA) cultured in two 10-cm culture dishes (6×10^6 cells/dish) for 16 hr were transfected with a mixture of a p16L1h plasmid (13.5 μg), a p16L2h plasmid (3 μg), and pYSEAP plasmid (13.5 μg) using Fugene HD (Rosch Diagnostics, Mannheim, Germany). After 60 h, pseudovirions (PVs) were purified from the cells, as described previously [Kondo et al., 2007], and their infectivity was estimated from the SEAP activity in culture media of infected cells using a colorimetric assay.

Neutralization Assay

The neutralization assay was performed as described previously [Ochi et al., 2008]. Briefly, 50 μl of serum was diluted with 50 μl of neutralization medium (DMEM [without phenol red], 10% FBS, 1% nonessential amino acids, 1% GlutaMax-I) containing an aliquot of the PV stock (400 pg of HPV 16 L1) giving an optical density of approximately 1.0 in the SEAP activity assay conditions and then incubated for 1 h at 4°C. The amount of PV used was in the linear range of the dose–response curve. The mixture was then inoculated onto 293FT cells that had been cultured in 96-well plates (1×10^4 cells/well) for 16 h. Culture media were harvested after 4.5 days at 37°C, and SEAP activity was determined. The neutralization titer is presented as the reciprocal of the maximum dilution of serum that reduced SEAP activity to half the level of that in the control samples.

Statistical Analysis

All time-to-event analyses were based on the actual date of visits. For regression or progression, time to event was measured from the date of the index visit (i.e., the first instance of an abnormal cytology result) to the date of the visit at which cytological transition to normal or to cervical intraepithelial neoplasia grade 3 was first detected. Women whose lesions persisted or who dropped out of the study were censored at their last recorded return visit dates. Patients who had only one negative colposcopy/cytology result before loss to follow-up were censored at the last date of positive Pap tests. Subjects who were biopsied were censored at the time of their biopsy, regardless of the biopsy results, to reduce the potential for interference by the biopsy procedure on estimates of time of regression. Cumulative probability of disease regression or progression was estimated using the Kaplan–Meier method and compared with a log-rank test, and the Cox regression model was used for statistical adjustments. Patient age (18–29, 30–39, or 40–54 years of age) and initial biopsy results (cervical intraepithelial neoplasia grade 1 or 2) were included in the multivariate model for adjustments. The χ^2 or Fisher's exact test was used to determine whither presence of HPV 16-specific neutralizing antibodies is significantly associated with risk of viral persistence. All analyses were carried out using the JMP 7.0J statistics packages (SAS Institute, Cary, NC). Two-sided P-values were calculated throughout and differences were considered significant for $P < 0.05$.

RESULTS

A total of 242 women with low-grade squamous intraepithelial lesions (206 with cervical intraepithelial neoplasia grade 1 and 36 with grade 2) were enrolled in this study. Mean age was 35.7 years (range 19–52 years). The total number of clinical visits was 3850 and the mean follow-up time was 46.2 months (median 43.2; range 6.8–84.9). During the follow-up period, 26 lesions progressed to cervical intraepithelial neoplasia grade 3, and 159 spontaneously regressed to normal cytology. No progression to invasive cancer was observed.

Detection of serum neutralizing antibodies against HPV 16 was strongly associated with the presence of viral DNA in the cervix (Table I), consistent with previous reports [Ochi et al., 2008]. The detection rate of HPV 16-specific neutralizing antibodies was much higher in women infected with HPV 16 than in those infected with other HPV types or those without any HPV DNA (59.5% vs. 12.5%, $P < 0.0001$ by χ^2 test). HPV 16-specific neutralizing antibodies were detected in 10.4% (19/182) of women with HPV strains other than HPV 16 and in 33.3% (6/18) of those without any HPV DNA. In the great majority of those with HPV 16 neutralizing antibodies, titers ranged between 40 and 320. High-titer (≥1,280) neutralizing sera were found in two women infected with HPV 16 and in one without any HPV DNA.

In those infected with HPV 16, a trend was observed for longer persistence of low-grade squamous intraepithelial lesions in HPV 16-specific neutralizing antibody carriers (median time to cytological regression: 23.8 months vs. 7.2 months, Fig. 1A), but the difference did not reach statistical significance ($P = 0.18$). Statistical adjustments for age and initial

TABLE I. The Association Between Cervical HPV Genotypes and Neutralizing Antibodies Against HPV 16

		Neutralizing antibodies against HPV 16							
		Neutralization		Neutralizing titer					
Cervical HPV genotype	N	Negative	Positive (%)	40	80	160	320	640	≥1,280
HPV 16 DNA positive	42	17	25 (59.5%)*	7	6	2	6	2	2
HPV 16 alone	34	13	21 (61.8%)	4	5	2	6	2	2
Multiply infected	8	4	4 (50.0%)	3	1	0	0	0	0
HPV 16 DNA-negative	200	175	25 (12.5%)*	4	8	6	2	3	1
Negative	18	12	6 (33.3%)	0	1	1	0	2	1
HPV 6	2	2	0 (0.0%)	0	0	0	0	0	0
HPV 18	9	8	1 (11.1%)	0	0	0	1	0	0
HPV 31	5	4	1 (20.0%)	0	1	0	0	0	0
HPV 33	2	1	1 (50.0%)	1	0	0	0	0	0
HPV 35	4	4	0 (0.0%)	0	0	0	0	0	0
HPV 39	4	4	0 (0.0%)	0	0	0	0	0	0
HPV 51	29	27	2 (6.9%)	1	1	0	0	0	0
HPV 52	35	31	4 (11.4%)	0	2	1	0	1	0
HPV 53	4	4	0 (0.0%)	0	0	0	0	0	0
HPV 56	23	22	1 (4.3%)	0	1	0	0	0	0
HPV 58	24	22	2 (8.3%)	1	1	0	0	0	0
HPV 59	2	1	1 (50.0%)	0	1	0	0	0	0
HPV 61	1	1	0 (0.0%)	0	0	0	0	0	0
HPV 66	6	5	1 (16.7%)	0	0	1	0	0	0
HPV 68	2	2	0 (0.0%)	0	0	0	0	0	0
Undetermined	14	12	2 (14.3%)	0	0	1	1	0	0
Multiple infection	16	13	3 (18.8%)	1	0	2	0	0	0

*The difference was statistically significant ($P < 0.0001$ by χ^2 test).

biopsy results (cervical intraepithelial neoplasia grade 1 or 2) did not change this finding (data not shown). Interestingly, progression to cervical precancer occurred only in those who had HPV 16-specific neutralizing antibodies at the baseline (Fig. 1B), and the associated risk of progression was statistically significant ($P = 0.03$ by log-rank test). Among women with cervical intraepithelial neoplasia grade 2 lesions, all (3/3, 100%) who had HPV 16-specific neutralizing antibodies at the baseline were diagnosed with grade 3 lesions within 5 years, while no such progression was observed in those who did not have HPV 16 neutralizing antibodies (0/5, 0%) ($P = 0.01$ by log-rank test). Among women with cervical intraepithelial neoplasia grade 1, 13.6% (3/22) positive for HPV 16 neutralizing antibodies were diagnosed with grade 3 disease within 5 years, while no such progression was found in those without HPV 16 neutralizing antibodies (0/12, 0%) ($P = 0.20$ by log-rank test). Adjusted P-values could not be calculated in the Cox proportional hazard model because no event occurred among women without serum HPV 16 neutralizing antibodies.

Neutralizing antibody responses resulting from previous HPV 16 clearance did not favor better outcomes of cytological abnormalities induced by other HPV types. The probability of disease regression within 2 years was not significantly different between women with or without serum HPV 16-specific neutralizing antibodies (44.0% vs. 57.0%, $P = 0.35$ by log-rank test). There was also no significant difference between these two groups in probability of progression to cervical intraepithelial neoplasia grade 3 within 5 years (8.3% vs. 12.0%, $P = 0.44$ by log-rank test). Analyses confined to the various cervical HPV 16-related types studied (HPV 31, HPV 33, HPV 35, HPV 52, and HPV 58) or statistical adjustment for age and initial biopsy results did not change these findings (data not shown).

HPV DNA data at baseline and 24 months were analyzed to determine whether detection of HPV 16-specific neutralizing antibodies was associated with persistent HPV infections. Results at 24 months were available for 149 women. Persistent infection was defined as continued detection at 24 months of HPV genotypes present at baseline. Among women infected with HPV 16 (n = 26), HPV 16 persistence was more common in those positive for serum HPV 16 neutralizing antibodies than in those with none (61.5% vs. 15.4%, Fisher's exact test: $P = 0.04$). Women who had serum HPV 16 neutralizing antibodies were found to be at a much higher risk of persistent infection compared with those who had none (odds ratio 8.06, 95% confidence interval 1.51–51.3). Among women infected with other HPV types (n = 123), HPV 16 neutralizing antibodies were not associated with persistent infections by baseline HPV genotypes (χ^2 test, $P = 0.61$).

DISCUSSION

In women with low-grade cervical lesions induced by HPV 16, progression to cervical precancer (cervical intraepithelial neoplasia grade 3) occurred only

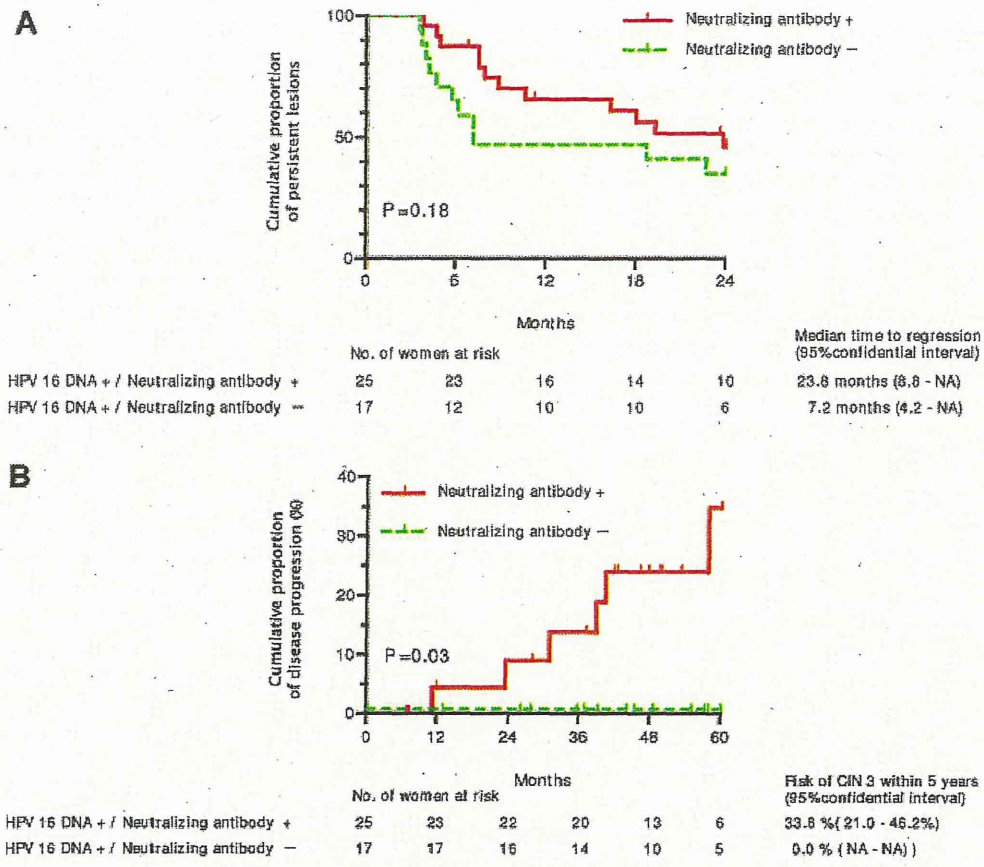

Fig. 1. Neutralizing Antibody against HPV 16 and Natural History of Low-grade Squamous Intraepithelial Lesion induced by HPV 16. Kaplan–Meier plots were used to estimate cumulative probabilities of regression (**A**) and progression (**B**) of low-grade squamous intraepithelial lesion induced by HPV 16 in relation to HPV 16 neutralizing antibody status. *P*-values calculated by log-rank test. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jmv]

among women carrying HPV 16-specific neutralizing antibodies. This finding is consistent with two previous reports of ELISA-based studies: HPV 16 VLP-specific antibodies were detected in all women whose disease eventually progressed to cervical precancer during follow-up, although results of statistical tests were not shown [de Gruijl et al., 1997; Sasagawa et al., 1998]. The use in this study of a neutralization assay which is more sensitive and type-specific than the VLP-based ELISA [Pastrana et al., 2004] demonstrated that the presence of neutralizing antibodies to HPV 16 confers a significantly higher risk of progression to cervical precancer among women with low-grade cervical abnormalities induced by HPV 16 ($P = 0.03$). The reason for this association may be that generation of antibodies to HPV 16 capsids reflects viral persistence. In the ELISA-based studies, HPV 16 capsid antibodies were more frequently detected in women who were persistently HPV 16 DNA-positive [de Gruijl et al., 1997; Sasagawa et al., 1998]. In the present study, low-grade cervical lesions were more likely to persist longer among women with HPV 16-specific neutralizing antibodies. These observations suggest that testing for VLP-specific or neutralizing antibodies to cervical HPV infection may identify women who are at high risk of viral persistence and disease progression. Although it was reported previously that type-specific HPV testing for women with low-grade cervical lesions is useful for identifying populations at increased risk of disease progression [Matsumoto et al., 2011], combined HPV typing and serological assays may aid more accurate stratification according to risk of disease persistence and progression.

An earlier study demonstrated that detection of HPV 16 neutralizing antibodies in sera was significantly associated with spontaneous regression of cervical intraepithelial neoplasia grade 1 lesions induced by HPV 16 [Kawana et al., 2002]. This result is contrary to the findings of the present study. The discrepancy may be explained in part by differences in the neutralization assays employed. The SEAP-mediated neutralization assay has been demonstrated to be more sensitive and type-specific than the assay used previously [Pastrana et al., 2004]. Indeed, the detection rate of HPV 16 neutralizing antibodies among

women with cervical intraepithelial neoplasia grade 1 induced by HPV 16 was much higher in the present than the previous study [Kawana et al., 2002] (64.7% vs. 21.6%). The present results are consistent with a recent report indicating that serum HPV 16 neutralizing antibodies were detected in 77.7% of women with cervical precursor lesions induced by HPV 16 [Mbulawa et al., 2008]. Furthermore, the present results were obtained from more extensive long-term follow-up data. In the present study, all cytological and histological data were reviewed by two cytopathologists and two pathologists. The median follow-up period was longer in this study compared to that of the previous report (46 vs. 24 months), which allowed for investigation of the association between HPV 16-specific neutralizing antibody and disease progression. The present findings are also supported by several previous ELISA-based studies of women with low-grade cervical lesions [de Gruijl et al., 1997; Sasagawa et al., 1998; Matsumoto et al., 2006] in which VLP-specific IgG antibodies were not associated with spontaneous regression of low-grade cervical abnormalities.

The presence of serum HPV 16-specific neutralizing antibodies in women with low-grade cervical lesions induced by other HPV types suggests that exposure to a given type may not protect against infections by other types and subsequent development of cervical lesions. In addition, the present study also demonstrated that neutralizing antibody responses to a given type did not influence the clinical outcomes of cervical precursor lesions induced by other types. This finding suggests that host adaptive immunity arising from previous viral clearance may not favor the clearance of low-grade cervical lesions induced by other types. This may be explained by several findings that epithelial cells expressing HPV antigens may not reactivate memory CTL due to impaired antigen presentation [Matsumoto et al., 2004; Wolkers et al., 2004]. Alternatively, immune responses inducing regression of low-grade cervical lesions may be HPV type-specific, which would have important implications for the design of therapeutic vaccines against cervical intraepithelial neoplasia [Kadish et al., 1997].

In the present study, the detection rate of HPV 16 neutralizing antibodies among women without HPV DNA was relatively high (33.3%, 6/18). These women did not have HPV 16 DNA by both PCR methods. Among the women without any HPV DNA, abnormal cervical cytology persisted for more than 24 months in 2 of 6 women having HPV 16 neutralizing antibodies but in none of 12 women lacking HPV 16 neutralizing antibodies ($P = 0.03$). Since detection of serum HPV 16 neutralizing antibodies strongly correlates with the presence of HPV 16 DNA in the cervix [Mbulawa et al., 2008; Ochi et al., 2008], the finding of HPV DNA negativity among some women positive for HPV 16 neutralizing antibodies might be due to sampling errors in the collection of cervical specimens.

VLP-based vaccination eliciting high titers of neutralizing antibodies have no therapeutic effect on an existing viral infection in animals and humans [Kirnbauer et al., 1996; Hildesheim et al., 2007]. Clearance rates of HPV 16 and HPV 18 infections at 6 and 12 months were similar between vaccinated and unvaccinated women, indicating that neutralizing antibodies elicited by VLP-based vaccination does not affect either viral clearance or persistence [Hildesheim et al., 2007]. In the present study, however, the HPV 16 neutralizing antibody response induced by natural infections was significantly associated with HPV 16 persistence. These observations suggest that HPV neutralizing antibodies induced by natural infection may be immunologically quite different from those elicited by vaccination.

To date, to the best of our knowledge, no measured immune response has been shown to define immunological control of established HPV infections. Available neutralization assays are useful for estimating protective immunity in vaccinated women. However, neutralizing antibodies elicited by current viral infection or previous clearance of other HPV types did not serve as a marker of the host's ability to control the viral infection and its associated cervical disease. The available serological assays provide only a partial characterization of host immune status and VLP-specific immune responses seem unrelated to the process of viral clearance.

In summary, a serum neutralizing antibody response against HPV 16 did not favor a better outcome for low-grade cervical lesions induced by HPV 16 or by other HPV types; rather, detection of neutralizing antibodies against cervical HPV may help identify women who are at high risk of viral persistence and disease progression. Although the present data suggest the potential usefulness of combined HPV DNA genotyping and type-specific neutralization assays in the management of women with low-grade cervical lesions, further studies are warranted to validate these results.

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