

# Immune responses against human papillomavirus (HPV) type 16 virus-like particles in a cohort study of women with cervical intraepithelial neoplasia

## II. Systemic but not local IgA responses correlate with clearance of HPV-16

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To investigate whether there is an association between local or systemic IgG and IgA responses against human papillomavirus (HPV) type 16 virus-like particles (VLP) containing L1 and L2 and the possible influence of these responses on clearance of HPV-16 and its associated lesions, cervical mucus samples from 125 patients and plasma samples from 100 patients, all participating in a non-intervention cohort study of women with abnormal cytology, were analysed. The results show that local IgG and IgA HPV-16 VLP-specific antibodies do not correlate with virus clearance. However, systemic IgG responses were more frequently detected in patients with a persistent infection (11/24) compared with patients with cleared HPV-16 infections (3/28,  $P = 0.006$ ). Furthermore, the ultimate development of high-grade lesions was associated with systemic VLP-specific IgG reactivity ( $P = 0.026$ ). By contrast, systemic IgA responses were correlated with virus clearance (7/28 clearance compared with 1/24 persistence patients,  $P = 0.06$ ). This correlation was statistically significant when only those clearance patients who tested HPV-16 DNA-positive at more than one visit were included in the analysis (5/11 compared with 1/24,  $P = 0.007$ ). As these systemic IgA responses were not accompanied by local IgA responses, the systemic IgA responses in HPV-16 clearance patients are suggested to be a by-product of a successful cellular immune response induced at the local lymph nodes, mediated by cytokines.

### Introduction

It has become clear from epidemiological and experimental studies that oncogenic human papillomaviruses (HPV), of which HPV-16 is the most common, play a crucial role in the development of cervical carcinoma (Bosch *et al.*, 1995; IARC,

1995). Failure of the immune response may contribute to the development of premalignant and malignant lesions, as is suggested by an increased incidence of HPV-associated lesions in immunocompromised hosts (Halpert *et al.*, 1986; Laga *et al.*, 1992). Furthermore, HPV infection and HPV-related premalignant lesions are characterized by high frequencies of spontaneous clearance and regression, respectively, suggesting a role for an immune response directed against HPV. We are currently investigating the role of HPV-16-specific immune responses in the course of HPV-induced cervical neoplasia and HPV-16 infection patterns, in a prospective non-intervention follow-up study of patients initially diagnosed with mild to

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moderate cervical dysplasia (Remmink *et al.*, 1995). We previously reported on the systemic IgG responses against HPV-16 virus-like particles (VLP) made up of L1/L2 in this patient group. HPV-16 VLP-specific IgG responses were detected in the majority of patients with a persistent HPV-16 infection and in patients with a high-grade cervical intra-epithelial neoplasia (CIN) lesion, as confirmed by histology at the end of follow-up. Since IgG responses were detectable only in a subset of patients with a cleared infection, the role of HPV-16 VLP-specific IgG antibodies with respect to the clearance of HPV-16 and premalignant lesions remains unclear (de Gruijl *et al.*, 1997). HPV-specific immune responses during the early phases of HPV infection may be important in blocking re-infection and preventing virus persistence. A recent study showed that HPV VLP type-specific IgA antibodies can be detected in the cervical mucus and are related to the presence of viral DNA (Wang *et al.*, 1996). Immunoglobulin subclass switches are under the control of various cytokines. Important sources of these cytokines are mucosal stratified epithelia (Schröder, 1995) and lymphoid cells. Consequently, studies of HPV-specific Ig isotypes may provide information about the role of humoral and cellular immune responses in HPV-associated disease.

This study was performed to investigate the relationship between local and systemic immunoglobulin responses (both IgA and IgG) against HPV-16 VLP and the possible influence of these responses on virus clearance and the behaviour of premalignant cervical lesions.

## Methods

**■ Patients.** The patients studied participated in a non-intervention cohort study of patients with cervical dysplasia ( $n = 352$ ) that was performed between 1990 and 1996. This study was designed and conducted to determine the relationship between HPV infection patterns and CIN disease course (Remmink *et al.*, 1995). Cervical mucus samples were collected between May 1992 and December 1994 and stored; plasma samples were collected from June 1993 until the end of follow-up. From this cohort, 125 patients were selected on the basis of availability of plasma at the end of follow-up and of cervical mucus. Patients presenting with abnormal cytology were enrolled in the study after giving written, informed consent. The study design was approved by the

ethics committee of the hospital. Patients entered the prospective study with mild to severe dyskaryosis. Details of collection and a description of clinical data have been reported in the preceding paper (de Gruijl *et al.*, 1999). At the end of follow-up, biopsies were taken for histological diagnosis (CIN 0, normal or metaplastic epithelium; CIN I, mild dysplasia; CIN II, moderate dysplasia; CIN III, severe dysplasia or carcinoma *in situ*). Details of the cohort study were described by Remmink *et al.* (1995). Patients with an HPV-16-positive smear at least once during follow-up (HPV-16<sup>+</sup>;  $n = 66$ ) were included in this study, as well as patients that had been HPV-negative during the complete follow-up of at least 12 months (HPV<sup>-</sup>,  $n = 31$ ; mean follow-up time, 35.8 months). The HPV-16<sup>+</sup> group was stratified according to HPV-16 infection status as previously defined (de Gruijl *et al.*, 1997). Briefly, patients with a persistent infection (HPV-16 P,  $n = 34$ ) were consistently positive for HPV-16 DNA over a period of at least 12 months (mean follow-up time, 39.2 months) and provided three consecutive PCR tests before Ig responses were measured. Patients showing HPV-16 clearance (HPV-16 C,  $n = 32$ ) were consistently negative for HPV-16 DNA over a period of at least 12 months (mean follow-up time, 46.4 months) and provided three consecutive PCR tests after having been HPV-16 DNA positive. Finally, a group of patients with a current or past infection of an HPV type other than 16 was included (HPV non-16,  $n = 28$ ; mean follow-up time, 47.2 months). This group consisted of 15 patients infected with an HPV type related to HPV-16 and 13 patients infected with an unrelated HPV type. For most patients at least two samples were analysed. Details of the number of samples tested in each group are given in Table 1.

**■ HPV detection and genotyping.** HPV detection and genotyping were performed on cellular material from cervical smears as described previously (de Roda Husman *et al.*, 1995; de Gruijl *et al.*, 1999).

**■ Preparation of cervical samples and plasma.** Cervical smears used for HPV typing were collected in 5 ml PBS/0.05% Merthiolate. After centrifugation, the pellets were used for HPV DNA testing and the supernatants were stored at  $-20^{\circ}\text{C}$  for IgA and IgG testing. Plasma derived from heparinized blood was diluted 1:1 in PBS containing 0.5% BSA and stored at  $-20^{\circ}\text{C}$ . Before use, plasma samples were centrifuged at 2300 *g* for 10 min at room temperature to remove protein debris.

**■ HPV-16 VLP ELISA.** Self-assembled HPV-16 L1/L2 VLP were expressed in baculovirus-infected Sf-9 insect cells, isolated and purified by caesium chloride gradient centrifugation as previously described (Kirnbauer *et al.*, 1993). These VLP were used in an ELISA to determine specific plasma and mucus IgG and IgA reactivity, by a modification of the assay reported previously by Kirnbauer *et al.* (1994). Approximately 300 ng or 50 ng per well VLP in PBS was added to 96-well ELISA plates (Maxisorp, Nunc) for cervical samples and plasma samples, respectively, and plates were incubated at  $37^{\circ}\text{C}$  for 1.5 h. Subsequently, the plates

**Table 1.** Number of patients and samples in the different patient groups

Group	Cervical samples		Plasma samples		Patient age (years)		Follow-up time (months)	
	No. patients	Total no. samples	No. patients	Total no. samples	Mean	Range	Mean	Range
HPV <sup>-</sup>	31	60	27	58	36.9	22–53	35.8	15–53
HPV-16 C	32	64	28	62	31.5	20–53	46.4	22–73
HPV-16 P	34	64	24	47	31.1	20–46	39.2	21–56
HPV non-16	28	49	21	38	32.2	16–52	47.2	25–72

**Table 2.** Systemic and local IgA and IgG positivity against HPV-16 VLP in relation to current HPV-16 DNA status

Test group	Local IgA		Local IgG		Systemic IgA		Systemic IgG	
	No.*	P†	No.	P	No.	P	No.	P
HPV DNA <sup>-</sup>	9/111 (8.1)	<b>0.04‡<sup>a</sup></b>	9/111 (8.1)	0.34	20/123 (16.3)	0.09	18/123 (14.6)	<b>0.002‡<sup>b</sup></b>
HPV-16 DNA <sup>+</sup>	13/65 (20.0)		9/65 (13.8)		2/44 (4.5)		17/44 (38.6)	

\* The number of patients in each group showing a response is shown as no. positive/no. tested. The percentage of positive responses is shown in parentheses.

† The significance of the presence of HPV-16 DNA was tested with a two-tailed  $\chi^2$  test.

‡ Odds ratios as follows: *a*, OR = 2.8 (1.1–7.4); *b*, OR = 3.7 (1.6–8.4).

were washed three times with PBS, blocked for 2 h at 37 °C with PBS containing 0.1% Tween 20 (Genfarma) (PBST) and 1% newborn calf serum (NBCS) (Gibco BRL) and again washed three times with PBS. The plates were then incubated with human plasma (diluted 1:10 in PBST–NBCS, 100  $\mu$ l per well) or cervical samples (undiluted, 100  $\mu$ l per well) for 2 h at room temperature with gentle rocking. After washing five times with PBS, 100  $\mu$ l HRP-labelled rabbit anti-human IgG or HRP-labelled rabbit anti-human IgA (Dako), both diluted 1:1000 in PBST–NBCS, was added per well. The plates were left for 1 h at room temperature and again washed five times with PBS. Finally, 0.2 g/ml *o*-phenylenediamine (Dako), dissolved in 0.64% KH<sub>2</sub>SO<sub>4</sub> plus 0.05% Na<sub>2</sub>HPO<sub>4</sub>·H<sub>2</sub>O (pH 5.4) containing 0.02% H<sub>2</sub>O<sub>2</sub>, was added (100  $\mu$ l per well). The reaction was stopped by the addition of 100  $\mu$ l 1 M H<sub>2</sub>SO<sub>4</sub> per well. Subsequently, the absorbance at 492 nm was measured with an ELISA plate reader (ICN). Duplicate tests were performed for all samples. The background reactivity found in negative-control wells coated with plain PBS and blocked with PBST–NBCS was subtracted from the absorbances obtained for the corresponding test wells of each sample. IgG and IgA reactivities were expressed as the mean absorbance of the duplicate tests. To compensate for inter-assay variability, absorbances were adjusted in relation to absorbances obtained for a set of internal control plasma samples (with both positive and negative IgG and IgA reactivities) that were included in each plate. The coefficient of variation of the reference plasma samples was 3.8% for IgA and 3.2% for IgG. IgG internal control reference sera were kindly provided by H. D. Strickler (NCI, Bethesda, USA).

To exclude the possibility that levels of VLP-specific IgA present in plasma would be artificially low due to antigenic competition in the presence of high amounts of VLP-specific IgG, IgG was absorbed with protein G from 12 plasma samples with high levels of VLP-specific IgG and low levels of VLP-specific IgA. No VLP-specific IgG was detectable after absorption and no differences were observed in VLP-specific IgA reactivity before and after IgG absorption (linear regression analysis;  $r = 0.99$ ,  $P < 0.0001$ ).

To investigate the comparability of the IgA detection method used here with the method previously used in other studies (Wang *et al.*, 1996; Elfgrén *et al.*, 1996), 90 cervical samples were analysed for HPV-16 VLP-specific IgA reactivity in the Department of Pathology, Free University Hospital, Amsterdam, by the method described above, and at the Microbiology and Tumor Biology Center, Karolinska Institute, Stockholm, by a method described previously (Wang *et al.*, 1996). Briefly, VLP were coated onto microtitre plates in PBS overnight at 4 °C. Following blocking with 10% goat serum in PBS (GS–PBS), plates were incubated with the cervical samples diluted 1:2 in GS–PBS. After washing with 0.5% Tween 20 in PBS, the plates were incubated with mouse anti-human

IgA diluted in GS–PBS. Subsequently the plates were washed and incubated with HRP-labelled goat anti-mouse IgG diluted in GS–PBS. The substrate, 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid), diammonium salt (ABTS), was added and after development absorbances at 405 nm were recorded. The  $A_{405}$  readings obtained in Stockholm were in general higher than the  $A_{492}$  results obtained in Amsterdam with identical samples. Therefore, the correlation coefficient was rather low ( $r = 0.48$ ) but the linear correlation between the results of the two methods was highly significant (linear regression analysis;  $P < 0.0001$ ).

The cut-off level, above which absorbances were considered positive, was based on the distribution of absorbances found in the HPV<sup>-</sup> patients. The cut-off values were determined by adding twice the SD to the mean absorbance after exclusion of the outliers (systemic IgA, 0.262; systemic IgG, 0.514; local IgA, 0.119; local IgG, 0.209).

■ **Statistical analysis.** Proportional analysis between patient groups was performed with the  $\chi^2$  test or Fisher's exact test (2 × 2 table analysis). Ig levels of samples taken at individual time-points during follow-up were compared by linear regression analysis. For direct comparison of  $A_{492}$  values between groups, the Mann–Whitney U (MWU) test was employed. All tests were two-tailed and considered significant when  $P < 0.05$ .

## Results

### IgA and IgG reactivity in relation to current HPV-16 DNA status

In all samples tested from HPV<sup>-</sup> and HPV-16<sup>+</sup> patients, local and systemic IgA and IgG reactivities were compared when cervical smears contained either HPV-16 DNA (local,  $n = 65$ ; systemic,  $n = 44$ ) or no HPV DNA at all (local,  $n = 111$ ; systemic,  $n = 123$ ). Twelve cervical samples from the HPV-16<sup>+</sup> group (from patients infected with other HPV types as well as with HPV-16) were excluded from this analysis because HPV types other than HPV-16 were detected in the smear at the time of testing. This analysis showed that local IgA responses were more frequently detected in the cervical mucus when HPV-16 DNA was detected in the smear [20.0% compared with 8.1%;  $P = 0.04$ , odds ratio, OR = 2.8 (95% confidence limits 1.1–7.4)] (Table 2). Local IgG response frequencies were similar whether HPV-16 DNA was present (13.8%) or not (8.1%). The systemic IgA response frequency correlated inversely with HPV-16 DNA presence in the smear

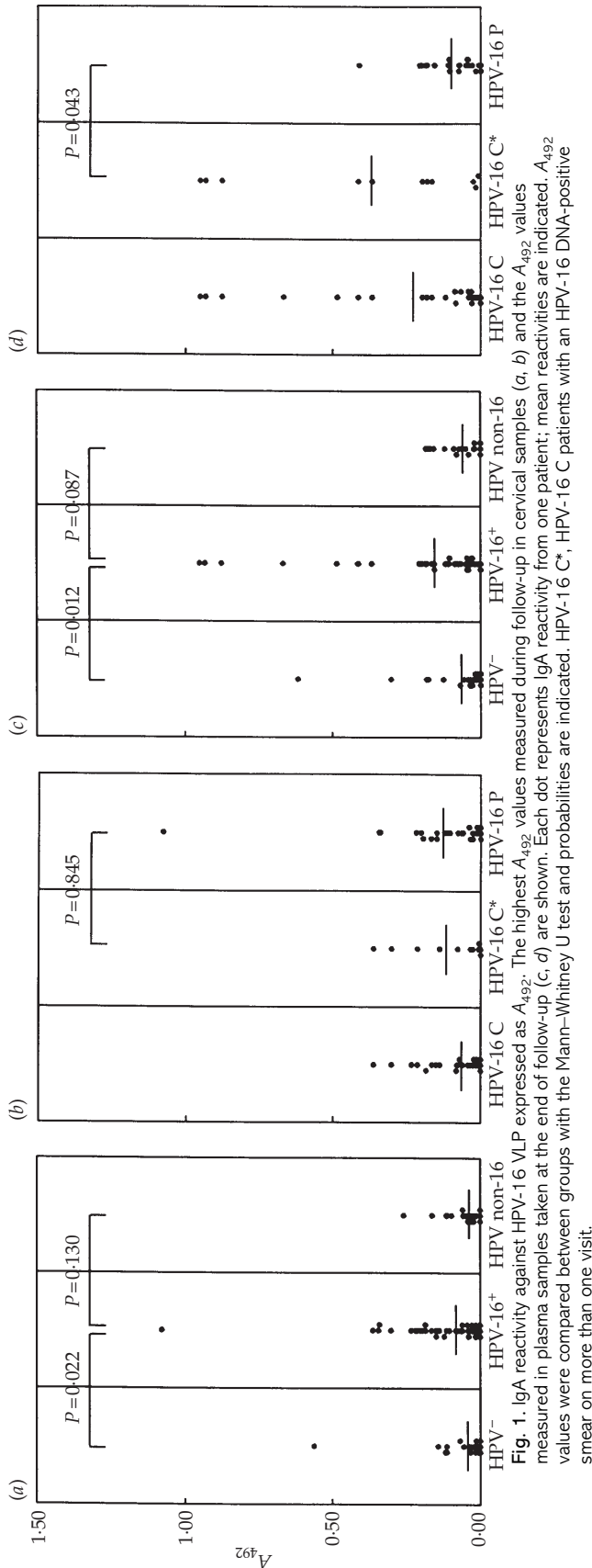


Fig. 1. IgA reactivity against HPV-16 VLP expressed as  $A_{492}$ . The highest  $A_{492}$  values measured during follow-up in cervical samples (a, b) and the  $A_{492}$  values measured in plasma samples taken at the end of follow-up (c, d) are shown. Each dot represents IgA reactivity from one patient; mean reactivities are indicated.  $A_{492}$  values were compared between groups with the Mann-Whitney U test and probabilities are indicated. HPV-16 C patients with an HPV-16 DNA-positive smear on more than one visit.

(16.3% compared with 4.5%, not significant; Table 2), while systemic IgG response frequencies correlated with HPV-16 DNA presence [38.6% compared with 14.6%;  $P = 0.002$ , OR = 3.7 (1.6–8.4)] (Table 2).

**IgA and IgG reactivity in relation to past or current HPV status**

Cervical samples were available at the start or end of follow-up for only a limited number of patients. Therefore, analysis could not be performed on the basis of a single time-point (i.e. at the start or end of follow-up). Moreover, because mucus antibody levels can fluctuate considerably during the reproductive cycle, analyses were performed based on the highest  $A_{492}$  value measured in cervical samples during follow-up for each patient. HPV-16 VLP-specific IgA reactivity in the cervical samples was significantly higher in the HPV-16<sup>+</sup> group compared with the HPV<sup>-</sup> group (Fig. 1a; MWU,  $P = 0.022$ ). No significant differences were observed between the HPV-16<sup>+</sup> and HPV non-16 groups (Fig. 1a; MWU,  $P = 0.13$ ). There was no significant difference in reactivity between the HPV-16 C and HPV-16 P groups (MWU,  $P = 0.944$ ), nor was there a significant difference when this analysis was performed on the basis of HPV-16 C patients who had an HPV-16 DNA-positive smear on more than one occasion (Fig. 1b; MWU,  $P = 0.845$ ).

HPV-16 VLP IgG reactivities were comparable between the HPV-16<sup>+</sup> (mean  $A_{492}$ , 0.095) and HPV<sup>-</sup> (mean  $A_{492}$ , 0.073) groups (MWU,  $P = 0.485$ ) and between the HPV-16 C (mean  $A_{492}$ , 0.077) and HPV-16 P (mean  $A_{492}$ , 0.159) groups (MWU,  $P = 0.207$ ).

Because plasma samples were available at the end of follow-up from the majority of patients included in this study, cross-sectional analysis of systemic Ig responses was performed at the end of follow-up. HPV-16 VLP-specific IgA reactivity in plasma was significantly higher in the HPV-16<sup>+</sup> group compared with the HPV<sup>-</sup> group (Fig. 1c; MWU,  $P = 0.012$ ) but the difference in systemic IgA reactivity between the HPV-16<sup>+</sup> group and the HPV non-16 group was not significant (Fig. 1c; MWU,  $P = 0.087$ ). There was no significant difference between the HPV-16 C and HPV-16 P groups (MWU,  $P = 0.883$ ). However, when this analysis included only those patients who tested positive for HPV-16 DNA on more than one visit, the IgA reactivity was significantly higher in the HPV-16 C group (Fig. 1d; MWU,  $P = 0.043$ ).

IgG reactivities tended to be higher in the HPV-16<sup>+</sup> group (mean  $A_{492}$ , 0.308) than in the HPV<sup>-</sup> group (mean  $A_{492}$ , 0.147) (MWU,  $P = 0.091$ ) and a significantly higher reactivity was detected in the HPV-16 P group (mean  $A_{492}$ , 0.457) compared with the HPV-16 C group (mean  $A_{492}$ , 0.180) (MWU,  $P = 0.006$ ).

Cut-off levels were calculated based on the distribution of IgA and IgG reactivities within the HPV<sup>-</sup> group (see Methods). Ig reactivities exceeding this value were considered positive.

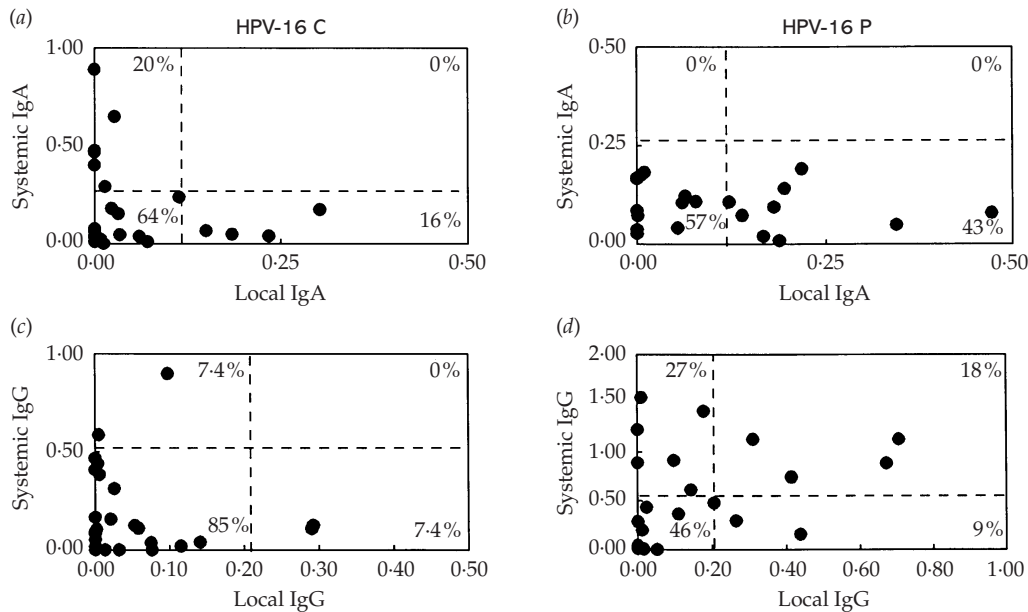
**Table 3.** Systemic and local IgA and IgG positivity against HPV-16 VLP in relation to HPV status

Test group	Local IgA		Local IgG		Systemic IgA		Systemic IgG	
	No.*	P†	No.	P	No.	P	No.	P
HPV <sup>-</sup>	2/31 (6.5)	<b>0.009</b> ‡ <sup>a</sup>	3/31 (9.7)	0.54	2/27 (7.4)	0.48	1/27 (3.7)	<b>0.014</b> ‡ <sup>b</sup>
HPV-16 <sup>+</sup>	20/66 (30.3)		11/66 (16.7)		8/52 (15.4)		14/52 (26.9)	
HPV-16 C	8/32 (25.0)	0.43	4/32 (12.5)	0.51	7/28 (25.0)	0.06	3/28 (10.7)	<b>0.006</b> ‡ <sup>c</sup>
HPV-16 P	12/34 (35.3)		7/34 (20.6)		1/24 (4.2)		11/24 (45.8)	
HPV non-16	2/28 (7.1)		4/28 (14.3)		0/21 (0.0)		3/21 (14.3)	
Related	2/15 (13.3)		3/15 (20.0)		NA		1/12 (8.3)	
Non-related	0/13 (0.0)		1/13 (7.7)		NA		2/9 (22.2)	

\* The number of patients in each group showing a response is shown as no. positive/no. tested. The percentage of positive responses is shown in parentheses. NA, Not applicable.

† Two-tailed  $\chi^2$  tests were carried out to test the significance of differences between the HPV<sup>-</sup> and HPV-16<sup>+</sup> groups and between the HPV-16 C and HPV-16 P groups.

‡ Odds ratios as follows: a, OR = 6.3 (1.4–29.1); b, OR = 9.6 (1.3–72.4); c, OR = 7.1 (1.6–32.4)



**Fig. 2.** Corresponding local and systemic responses measured in samples collected at the same visit during follow-up. IgA responses (a, b) and IgG responses (c, d) in HPV-16 C patients (a, c) and HPV-16 P patients (b, d) are shown. Cut-off values are indicated by the dotted lines.

Table 3 shows the frequencies of positive responses in all test groups. There was a significant difference between the HPV<sup>-</sup> and HPV-16<sup>+</sup> patients for the local IgA response [6.5% versus 30.3%;  $P = 0.009$ , OR = 6.3 (1.4–29.1)] (Table 3). There was no difference in response rates for local IgG between HPV<sup>-</sup> and HPV-16<sup>+</sup> patients (9.7% versus 16.7%,  $P = 0.54$ ) (Table 3). Both local IgA and IgG response frequencies were similar in the HPV-16 P and HPV-16 C patient groups (Table 3).

Systemic IgA responses were found in 25% of the HPV-16 C patients and in 4.2% of the HPV-16 P patients ( $P = 0.06$ , Table 3). This difference was significant when only those HPV-

16 C patients who had been HPV-16 DNA-positive at more than one visit were included [45.5% (5/11);  $P = 0.007$ , OR = 0.052 (0.005–0.5)]. The systemic IgG response frequencies correlated significantly with past or current HPV-16 infection [26.9% versus 3.7% in the HPV<sup>-</sup> patients;  $P = 0.014$ , OR = 9.6 (1.3–72.4)]. The IgG response frequency was significantly higher in the HPV-16 P group (45.8%) compared with the HPV-16 C group [10.7%;  $P = 0.006$ , OR = 7.1 (1.6–32.4)] (Table 3).

Responding patients in the HPV non-16 group were, or had been, infected with an HPV type related to HPV-16 in 2/2

**Table 4.** Systemic and local IgA and IgG positivity against HPV-16 VLP in relation to disease status in HPV-16<sup>+</sup> patients

Disease status of HPV-16<sup>+</sup> patients was assessed by cytology and colposcopy. CIN grades were allocated on the basis of colposcopy. The size of lesions was also assessed by colposcopy and the number of cervical quadrants the lesions occupied is shown.

Test group	Local IgA		Local IgG		Systemic IgA		Systemic IgG	
	No.*	P†	No.	P	No.	P	No.	P
<b>Cytology‡</b>								
Pap 1 and 2	13/77 (16.9)	0.96	8/77 (10.4)	0.74	15/85 (17.6)	0.47	15/85 (17.6)	<b>0.020</b> § <sup>a</sup>
Pap 3a and 3b	8/42 (19.0)		6/42 (14.3)		2/23 (8.7)		10/23 (43.5)	
<b>Colposcopy</b>								
CIN 0/I	13/79 (16.5)	0.97	6/79 (7.6)	0.21	17/80 (21.3)	<b>0.016</b>	13/80 (16.3)	<b>0.012</b> § <sup>b</sup>
CIN II/III	9/49 (18.4)		8/49 (16.3)		0/29 (0.0)		12/29 (41.4)	
0–2 quadrants	14/88 (15.9)	0.75	9/88 (10.2)	0.94	15/81 (18.5)	0.52	14/81 (17.3)	<b>0.032</b> § <sup>c</sup>
3–4 quadrants	8/40 (20.0)		5/40 (12.5)		2/28 (7.1)		11/28 (39.3)	

\* The number of patients in each group showing a response is shown as no. positive/no. tested. The percentage of positive responses is shown in parentheses.

† Two-tailed  $\chi^2$  tests were carried out to test the significance of differences between the Pap 1/2 and Pap 3a/3b groups, the CIN 0/I and CIN II/III groups, and the 0–2 quadrants and 3–4 quadrants groups.

‡ The cervical smears from nine samples could not be evaluated for cytology.

§ Odds ratios as follows: a, OR = 3.6 (1.2–10.6); b, OR = 3.6 (1.3–9.8); c, OR = 3.1 (1.1–8.8).

cases for local IgA, 3/4 for local IgG and 1/3 for systemic IgG. None of the HPV non-16 patients showed positive systemic IgA responses (Table 3).

### Correlation between local and systemic Ig responses

Regression analysis showed a positive correlation between local and systemic IgG responses at 49 individual time-points in the HPV-16<sup>+</sup> patient groups (linear regression analysis;  $r = 0.297$ ,  $P = 0.025$ ). The correlation coefficient is low because the  $A_{492}$  readings of the cervical samples were generally lower than the  $A_{492}$  readings of the plasma samples. There was no correlation between local and systemic IgA responses (linear regression analysis;  $r = 0.055$ ,  $P = 0.682$ ) in these patient groups.

Fig. 2 illustrates the relationship between local and systemic  $A_{492}$  values measured in samples collected at the same visit during follow-up for both IgA (Fig. 2*a, b*) and IgG (Fig. 2*c, d*) in HPV-16 C (Fig. 2*a, c*) and HPV-16 P (Fig. 2*b, d*) patients. Positive IgA responses were never detected at the same time in cervical samples and plasma (Fig. 2*a, b*). The same holds true for IgG responses in HPV-16 C patients (Fig. 2*c*). However, in the HPV-16 P patient group, 18% of the matched samples tested were positive for both systemic and local IgG (Fig. 2*d*).

### IgA and IgG reactivities in relation to disease course during follow-up

Table 4 shows the results of the proportional analysis of Ig levels measured in all samples during follow-up of the HPV-16 DNA<sup>+</sup> patients. The proportion of cervical samples that

showed local IgA and IgG reactivity was similar in the groups with normal cytology and abnormal cytology and in the groups with small or low-grade and large or high-grade lesions, as defined by colposcopy during follow-up (Table 4). Abnormal smears and more severe and larger lesions, as determined by colposcopy, were associated with systemic IgG responses (Table 4). Systemic IgA response frequencies were comparable when the smears showed normal or abnormal cytology. However, no positive systemic IgA responses were found in conjunction with high-grade colposcopic CIN, compared to a response frequency of 21.3% in conjunction with low-grade colposcopic CIN ( $P = 0.016$ , Table 4). IgA responses were independent of the size of the lesion (Table 4).

### IgA and IgG reactivities in relation to histological grade at the end of follow-up

Analysis based on the highest  $A_{492}$  value measured during follow-up revealed that local IgA and IgG response frequencies were similar in patients with histologically confirmed low- or high-grade lesions at the end of follow-up (Table 5).

Proportional analysis of systemic responses at the end of follow-up revealed a higher IgG response frequency in patients with histologically confirmed CIN III lesions compared with patients diagnosed with lesions less severe than CIN III [53.8% versus 17.9%, OR = 5.3 (1.2–24.0),  $P = 0.026$ ] (Table 5). The opposite was seen for systemic IgA responses, although the difference was not significant; 20.5% of responding patients with a lesion less than CIN III compared with none of the patients who developed a CIN III lesion ( $P = 0.18$ , Table 5).

**Table 5.** Systemic and local IgA and IgG positivity against HPV-16 VLP in relation to histological grade at the end of follow-up

Test group	Local IgA		Local IgG		Systemic IgA		Systemic IgG	
	No.*	P†	No.	P	No.	P	No.	P
CIN < III	12/41 (29.3)	1.00	5/41 (12.2)	0.30	8/39 (20.5)	0.18	7/39 (17.9)	<b>0.026‡</b>
CIN III	8/25 (32.0)		6/25 (24.0)		0/13 (0.0)		7/13 (53.8)	

\* The number of patients in each group showing a response is shown as no. positive/no. tested. The percentage of positive responses is shown in parentheses.

† Two-tailed Fisher's exact tests were carried out to test the significance of differences between the CIN < III and CIN III groups.

‡ OR = 5.3 (1.2–24.0).

## Discussion

We have analysed local and systemic HPV-16 VLP-specific IgA and IgG responses in patients with abnormal cytology. Mucus antibody levels can fluctuate considerably during the reproductive cycle (Van Ginkel *et al.*, 1997). We therefore measured the total IgA and IgG levels by ELISA in all cervical samples tested and corrected the VLP-specific  $A_{492}$  values for the total amount of IgA and IgG present (not shown). Analysis based on the corrected and uncorrected  $A_{492}$  values resulted in similar response rates among the different patient groups, so the uncorrected values were used in all analyses. Furthermore, positivity for HPV-16-specific local IgA and IgG responses was determined for most patients on the basis of two or more tested cervical samples.

The mean age of the HPV<sup>-</sup> patients was significantly higher than that of the HPV-16<sup>+</sup> patients (Table 1; MWU,  $P = 0.001$ ), which is in agreement with previously published data showing that the prevalence of cervical HPV infection declines with increasing age (Melkert *et al.*, 1993).

Regression analysis revealed that there was no correlation between local and systemic HPV-16 VLP-specific IgA levels measured in samples taken at individual time-points, but a good correlation between local and systemic IgG levels. It is therefore likely that the VLP-specific IgG antibodies that we detected locally had entered the mucosal surface by transudation from the serum, in contrast to the local IgA antibodies. This is also illustrated by Fig. 2, which shows that IgA responses were never detected simultaneously at the cervix and the periphery, while IgG responses were often found both locally and systemically at the same time in HPV-16 P patients. In some cases, local but not systemic IgG was detected, indicating that in these individuals some local IgG-producing B cells must be present.

In agreement with previous studies, local HPV-16 VLP-specific IgA (Wang *et al.*, 1996) and systemic IgG responses (Kimbauer *et al.*, 1994; Wideroff *et al.*, 1995; Roden *et al.*, 1996; Dillner *et al.*, 1996) correlated with the presence of HPV-16 in

the smear. We have previously shown that systemic IgG responses to HPV-16 VLP, containing either L1 and L2 (de Gruijl *et al.*, 1997) or L1 only (de Gruijl *et al.*, 1999), were associated with a persistent HPV-16 infection and were predominantly present in patients who developed a CIN III lesion. In this study this is reconfirmed in a slightly different stratified patient group from the same cohort. However, the percentage of patients responding in this study is slightly lower than in the previous published study (de Gruijl *et al.*, 1997) due to the higher cut-off points used. The cut-off values were determined on the basis of different populations, i.e. HPV<sup>-</sup> patients from the prospective study cohort in this study and a group of healthy donors in the previous study.

Local IgA and IgG did not correlate with virus clearance, nor was there any correlation with disease course. Therefore, it appears that mucosal virion-specific antibodies are not effective in inducing regression of established lesions. However, in contrast to previously published studies that showed a correlation between systemic IgA directed against VLP and squamous intraepithelial lesions (SIL) (Strickler *et al.*, 1997; Sasagawa *et al.*, 1998), we show that systemic IgA levels are correlated with virus clearance and resolution of the lesion. This discrepancy is probably due to different stratified control groups, which resulted in the use of lower cut-off levels in these other studies. The control patients in these other studies had no history of SIL and tested repeatedly negative for HPV DNA, while our control group consisted of patients who tested negative for HPV DNA on at least three separate occasions but did have a history of abnormal cytology. In the present study, positive systemic IgA responses could be detected in 25% of the HPV-16 C patients while only one of the HPV-16 P patients showed positivity (Table 3,  $P = 0.06$ ). As shown previously for systemic IgG responses (de Gruijl *et al.*, 1997), HPV-16 C patients who had been positive for HPV-16 DNA at two or more time-points showed a higher frequency of positive systemic HPV-16 VLP-specific IgA responses (45.5%) than did patients that had been positive at only one time-point (11.8%,  $P = 0.08$ ; not shown). The

correlation of IgA reactivity with clearance was significant in members of the HPV-16 C group who had an HPV DNA-positive smear on more than one visit ( $P = 0.007$ ), indicating that HPV-16 infection must be sustained for at least 6 months to allow VLP-specific IgA responses to be elicited. Sasagawa *et al.* (1998) reported an association of VLP-specific IgA with virus persistence. However, the persistence patients in their study were defined as having had at least two subsequent positive HPV DNA tests, while 9/10 of these persistence patients were HPV-16 DNA-negative at the end of follow-up. These patients might therefore be more comparable with our clearance patients (HPV-16 C).

Cervical samples taken at the same visit as the plasma samples were tested for 6/7 of the responding HPV-16 C patients and a positive local IgA response was not observed in any of these six patients. This might be explained by a decline of the local IgA response preceding the decline of the systemic responses. This is in agreement with previously published results, which showed that local VLP-specific IgA declines rapidly after successful treatment and elimination of HPV, whereas systemic VLP-specific IgA was more stable, despite virus elimination (Elfgren *et al.*, 1996).

Alternatively, the systemic IgA responses we detected may be a secondary marker of a successful immune response, mediated by cytokines and induced at the draining lymph nodes rather than by the local mucosae. As systemic IgA correlates with clearance of HPV-16, transforming growth factor- $\beta$  (TGF- $\beta$ ), which is the Ig class switching factor for IgA (Stavnezer, 1995), might promote virus clearance and regression of the lesion. Indeed, TGF- $\beta$  inhibits proliferation of both normal and HPV-infected keratinocytes (Braun *et al.*, 1990; Ho *et al.*, 1994). Therefore, autocrine control of keratinocyte proliferation by TGF- $\beta$ , especially at the early stages of HPV infection before the transformed keratinocytes become resistant to TGF- $\beta$ , might limit the infection and might possibly allow other cytokines produced by activated T cells, such as interferon- $\gamma$ , to clear the virus (Woodworth *et al.*, 1992). The release of virions will induce an antibody response that will switch to IgA under the influence of TGF- $\beta$  at the local lymph node.

In conclusion, naturally occurring HPV-16 VLP-specific local IgG and IgA responses do not appear to be correlated with HPV-16 clearance or persistence, or with regression or progression of the lesion. Local HPV-16 VLP-specific IgA antibodies appear to be produced locally, while local HPV-16 VLP-specific IgG antibodies appear to originate from the periphery. Systemic IgG responses were associated with persistent infection and the development of high-grade lesions, while systemic IgA responses were associated with virus clearance. These systemic IgA responses might be an indication of a successful cellular immune response mediated by cytokines.

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