

Detection of Human Papillomavirus Type 31-Neutralizing Antibodies from Naturally Infected Patients by an Assay Based on Intracellular Assembly of Luciferase-Expressing Pseudovirions[∇]

Maxime J. J. Fleury,^{1,2,3} Antoine Touzé,^{1,2,3} Silvia de Sanjosé,⁴ F. Xavier Bosch,⁴
Joellen Klaustermeier,⁴ and Pierre Coursaget^{1,2,3*}

INSERM U618, Tours, France¹; University François Rabelais, Tours, France²; IFR 136, Agents Transmissibles et Infectiologie, Faculté des Sciences Pharmaceutiques, Tours, France³; and Servei d'Epidemiologia i Registre del Cancer, Institut Catala d'Oncologia, Barcelona, Spain⁴

Received 4 July 2007/Returned for modification 28 August 2007/Accepted 29 October 2007

The aim of this study was to develop a highly sensitive human papillomavirus type 31 (HPV31) neutralization assay based on the production of pseudovirions carrying luciferase. Neutralizing antibodies against HPV31 were investigated in a set of HPV31 monoclonal antibodies and in women with evidence of HPV31 infection. Neutralizing antibodies were detected in 78% of subjects with a positive enzyme-linked immunosorbent assay.

Serologic studies have demonstrated that infection with genital human papillomaviruses (HPVs) is followed by a humoral immune response to viral capsid proteins and that anti-virus-like-particle (anti-VLP) antibodies can be an indicator of past as well as current infection (4, 7) and can persist for many years (1, 4, 23). Among subjects who had had an incident of infection with HPV type 16 (HPV16), seroconversion was a very slow process that required sufficient exposure (high viral load or persistence of infection), and fewer than two-thirds of infected women became seropositive (11). Whether this anti-VLP response elicited by natural HPV infection confers protection against reinfection with the same or related types remains a matter of discussion. Studies on neutralizing antibodies against HPVs have been hampered by the lack of a convenient cell culture system for the production of HPV virions and for monitoring HPV infections. Various methods have been developed to overcome this problem, based on neutralization of authentic virions (5, 16), pseudotyped virions (15), VLPs that have encapsidated reporter genes in cellular or cell-free systems (17, 18, 21), or VLPs expressing a reporter gene on their surfaces (2). Due to low production of pseudovirions, most of these assays lack sensitivity and are not reliable for detecting serum-neutralizing antibodies following natural infection, with the exception of the system recently described by Buck et al. (3) for the generation of high levels of pseudovirions and applied to the production of HPV pseudovirions for types 6, 16, 18, 31, 45, 52, and 58 (3, 9, 12, 14).

We report here a technique based on the production of pseudovirions obtained by cellular encapsidation of a plasmid coding for luciferase as a reporter for the development of a papillomavirus neutralization assay for HPV31. Validation of this assay was achieved by analyzing the neutralizing activity of

HPV31 monoclonal antibodies (MAbs) and sera from women naturally infected with HPV31. Two systems were used for the generation of pseudovirions. One was an acellular system of production, based on the direct interaction between HPV31 L1- and L2-expressing VLPs produced in *Sf21* insect cells and DNA coding for luciferase (2). The second method was based on the production of HPV31 pseudovirions in 293FT cells, using codon-modified HPV31 L1 and L2 genes. To increase the expression level of the HPV31 capsid proteins in mammalian cells, *Homo sapiens* codon-optimized HPV31 L1 (L1h) and HPV31 L2 (L2h) genes (GenBank database entries EU127831 and EU127832, respectively), with deletion of the internal polyadenylation signals and the addition of a Kozak sequence, were synthesized (Geneart, Regensburg, Germany) and cloned into the bicistronic expression vector pIRES, which contains the simian virus 40 ori (BD Biosciences, Clontech, France). Wild-type HPV31 L1 and L2 genes were cloned into the same vector as controls. Both constructs (pIRES31L1L2h and pIRES31L1L2wt; 9.0 kb) were transfected into 293FT cells by using Fugene6 according to the manufacturer's instructions, and nuclear fractions were analyzed by Western blotting, using H31.D24 MAb (8) and an L2 polyclonal antibody for L1 and L2 detection, respectively. The levels of expression of the L1 and L2 proteins were estimated by integration (Molecular Analyst software; Bio-Rad, Marnes-la-Coquette, France) of the Western blotting signals, and the amounts of L1 and L2 proteins produced with the optimized genes were 17 times higher than those produced with the wild-type genes (Fig. 1). HPV31 pseudovirions were produced by cotransfecting 293FT cells with pIRES31L1L2h and the pGL3 luciferase reporter plasmid (a 5.3-kbp plasmid containing the simian virus 40 ori). Cells were transfected with DNA and Fugene6 (Roche) and harvested at 2 days posttransfection for this purpose. Nuclear fractions, obtained as previously described (19), were loaded on a CsCl gradient and ultracentrifuged in a Beckman SW28 rotor (22 h at 27,000 rpm and 4°C). L1-positive gradient fractions were detected by Western blotting at densities of 1.35 to

* Corresponding author. Mailing address: Laboratoire Vecteurs, Virus & Vaccins, INSERM U618, Faculté des Sciences Pharmaceutiques, 31 Ave. Monge, 37200 Tours, France. Phone: 33 2 47 36 72 56. Fax: 33 2 47 36 71 88. E-mail: coursaget@univ-tours.fr.

[∇] Published ahead of print on 7 November 2007.

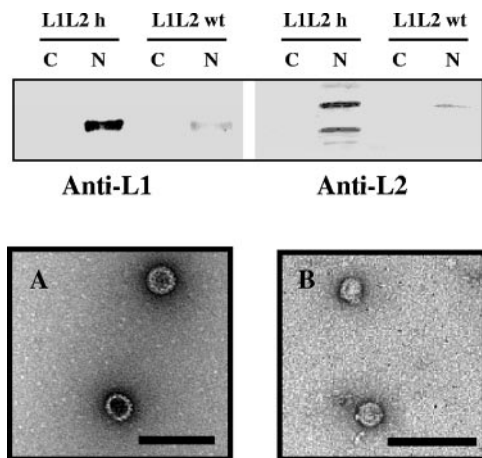


FIG. 1. Expression of wild-type (wt) and codon-optimized (h) HPV31 capsid protein level in human 293FT cells transfected with 10 μ g wild-type L1L2 (pIRES31L1L2wt) and codon-optimized L1L2 (pIRES31L1L2h) plasmids. L1 proteins were detected using H31.D24 MAb, and L2 proteins were detected by HPV18 L2 polyclonal rabbit serum. HPV31 L1L2h VLPs (A) and HPV31 L1L2h/pGL3 pseudovirions (B) were observed by transmission electron microscopy. Bars, 100 nm. Lanes C, cytoplasmic fraction; lanes N, nuclear fraction.

1.36, corresponding to the density of virions, whereas the L1-positive fractions from 293FT cells transfected with pIRES31L1L2h in the absence of the pGL3 luciferase reporter plasmid were detected only at densities of 1.26 to 1.27, corresponding to VLPs (data not shown). L1-reactive fractions were observed by electron microscopy on a JEOL 1010 electron microscope after being negatively stained (19), and the presence of pseudovirions and VLPs was confirmed (Fig. 1). The total amounts of VLPs present in the two pseudovirion preparations were estimated by enzyme-linked immunosorbent assay (ELISA) and Western blotting using anti-L1 antibody. The results indicated 20 to 25 times higher levels of L1 in the preparation obtained by the direct interaction method than those in the preparation obtained with 293FT cells. This suggests that the proportion of effective pseudovirions among the total VLPs produced using the direct interaction method is low, in accordance with the detection of L1 reactivity in CsCl gradients mainly at densities of 1.26 to 1.28 (corresponding to VLPs) and in contrast to the L1 reactivity detected mainly at densities of 1.35 to 1.36 (corresponding to the density of pseudovirions) with pseudovirions produced in 293FT cells (data not shown).

The ability of HPV31 L1L2h/pGL3 pseudovirions to transfer genes was investigated with COS-7 cells and compared to gene transfer with pseudovirions obtained by the direct interaction method. The two pseudovirion preparations were derived from the same number of producing cells. Luciferase expression of about 10^5 cps was observed at a 1:25 dilution of the crude HPV31 L1L2h pseudovirion stock, and $>10^3$ cps was observed at a dilution of 1:800 (Fig. 2). This was eight times higher than the gene transfer level observed with pseudovirions produced by the direct interaction method. To investigate whether the HPV31 L1L2h pseudovirion assay represented a substantial improvement over our previous neutralization assay, we inves-

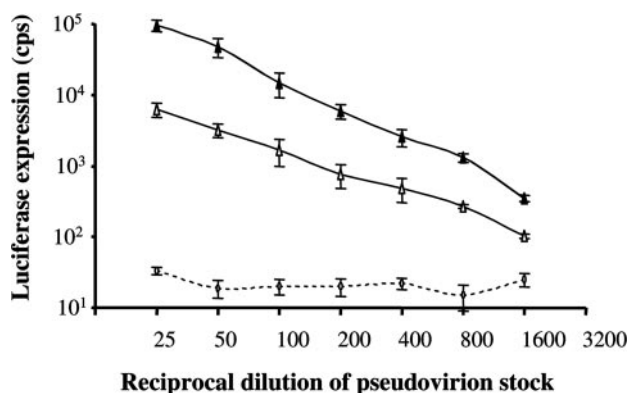


FIG. 2. Luciferase expression in COS-7 cells after infection with pseudovirions obtained by direct interaction between plasmid and VLPs or with pseudovirions obtained by intracellular encapsidation of the reporter gene in a capsid expressing humanized L1 and L2 genes. \blacktriangle , L1L2h pseudovirions; \triangle , L1L2 pseudovirions; \diamond , pLuc alone.

tigated 18 HPV31 MAbs (8) for neutralizing activity and compared the findings using a neutralization assay with pseudovirions obtained by the direct interaction method (Table 1). In these assays, the HPV31 pseudovirions were preincubated with antibodies, and then the inhibition in gene transfer expression was measured in COS-7 cells. The cutoff value used for these assays was inhibition of gene transfer of $>80\%$, a value previ-

TABLE 1. Neutralizing titers of HPV31 MAbs^a

MAb	ELISA titer (mean \pm SD)	Neutralizing titer (mean \pm SD)	
		Method A	Method B
CamVir-1	$>7,290$	0 ± 0	0 ± 0
H31.B5	30 ± 0	0 ± 0	0 ± 0
H31.E22	25 ± 7	0 ± 0	0 ± 0
H31.D24	$7,290 \pm 0$	0 ± 0	0 ± 0
H31.C19	$2,430 \pm 0$	0 ± 0	0 ± 0
H31.E17	25 ± 3	10 ± 0	30 ± 0
H31.B18	75 ± 22	30 ± 0	90 ± 0
H31.D7	810 ± 0	3 ± 0	30 ± 0
H31.F16	810 ± 0	90 ± 0	675 ± 202
H31.C24	$2,430 \pm 0$	15 ± 7	270 ± 0
H31.E16	$2,025 \pm 608$	45 ± 22	810 ± 0
H31.H9	$2,430 \pm 0$	90 ± 0	810 ± 0
H31.H17	$2,430 \pm 0$	90 ± 0	$2,430 \pm 0$
H31.F7	$7,290 \pm 0$	10 ± 0	30 ± 0
H31.A19	$7,290 \pm 0$	15 ± 7	270 ± 0
H31.B1	$7,290 \pm 0$	90 ± 0	810 ± 0
H31.G5	$7,290 \pm 0$	135 ± 67	$2,430 \pm 0$
H31.D14	$7,290 \pm 0$	90 ± 0	$3,645 \pm 1822$
H31.H12	$7,290 \pm 0$	810 ± 0	$7,290 \pm 0$

^aTiters were obtained by inhibition of luciferase gene transfer, using pseudovirions obtained by direct interaction of wild-type L1 and L2 VLPs with plasmid DNA and by encapsidation of the same plasmid within 293FT cells in which humanized L1 and L2 were expressed. The first 3 MAbs recognize linear epitopes, and the 15 others recognize conformational epitopes (8). Method A, pseudovirions obtained by direct interaction of wild-type L1 and L2 VLPs; method B, pseudovirions obtained by encapsidation with humanized L1 and L2 in 293FT cells. The ELISA end-point titer was determined as the reciprocal of the highest serum dilution giving an absorbance value greater than the average absorbance for sera from 30 infants plus three times the standard deviation (value of 0.200). The neutralizing end-point titer was determined as the reciprocal of the highest serum dilution giving $>80\%$ inhibition of luciferase expression. Data are means of four determinations for samples serially diluted threefold from 1/3 to 1/7,290.

TABLE 2. Detection of HPV31 neutralizing antibodies after natural infection ($n = 123$ subjects)

ELISA result	Population group (no. of patients)	ELISA antibodies		No. (%) of patient samples with neutralizing activity	Neutralizing antibodies	
		Median titer (95% CI)	Geometric mean titer (95% CI) ^c		Median titer (95% CI)	Geometric mean titer ^b (95% CI) ^d
Anti-HPV31 negative	All (68)			0 (0)		
	Virgins (20)			0 (0)		
	Sexually active women (29)			0 (0)		
	Prostitutes (19)			0 (0)		
Anti-HPV31 positive	All (55)	50 (42.8–64.4)	66.0 (49.6–87.8)	43 (78)	100 (50–200)	133.7 (85.7–208.5)
	Virgins (2)	25 (25–25)	25 (25–25)	2 (100)	25 (25–25)	25 (25–25)
	Sexually active women (34)	50 (25–50)	61.3 (42.6–88.3)	24 (71)	100 (50–200)	112.2 (66.6–189.3)
	Prostitutes (19)	100 (25–200)	83.3 (49.5–140.2)	17 (89)	100 (50–400)	208.3 (89.9–482.7)

^a ELISA and neutralization end-point titers are means of two to four determinations for serum samples serially diluted twofold from 1/25 to 1/3,200.

^b Calculated for neutralizing antibody-positive subjects.

^c The P value, based on log-transformed antibody titers, for subjects, excluding virgins, was 0.31.

^d The P value for subjects, excluding virgins, was 0.18.

ously determined using sera (diluted 1/20) from 20 infants and 10 nonimmunized mice that demonstrated <80% inhibition of luciferase expression. The results indicated that the same MAbs were neutralizing in both assays. However, the neutralizing titers were 3 to 27 times higher (mean, 15 times) with the neutralization assay using the L1L2h pseudovirions produced in 293FT cells than with the assay using pseudovirions obtained by the direct interaction method. The greater sensitivity of the test using pseudovirions generated in 293FT cells using optimized L1 and L2 genes may have been related to a greater ratio between pseudovirions and VLPs, since empty VLPs (noninfectious particles) can compete with pseudovirions for binding to neutralizing antibodies. In addition, it should be noted that in comparing the neutralizing titers and the ELISA titers, some MAbs were highly neutralizing, since their ELISA and neutralizing titers were similar (B18, E17, F16, H12, and H17), in contrast with others, such as A19, D7, B1, and F7, that evidenced low neutralizing capacities.

Sera from 68 anti-HPV31-negative and 55 anti-HPV31-positive women were also investigated for neutralizing antibodies (Table 2). These subjects were extracted from a larger study including practicing prostitutes and women from the general population recruited in Oviedo and Barcelona, Spain. Serum samples from 38 practicing prostitutes and 85 women from the general population (22 virgins and 63 sexually active women) were selected for this study on the basis of previous results of HPV DNA and anti-HPV31 VLP antibody detection (20). HPV31 DNA was detected in four women, two of whom were anti-HPV31 positive by ELISA. None of the anti-HPV31-negative samples in ELISA were found to be positive for anti-HPV31 by the neutralization assay. Data concerning neutralizing antibodies were log transformed, resulting in a normal distribution of the data, and analyzed as continuous variables by using geometric means. The chi-square test was used to evaluate associations between categorical variables and neutralizing antibody status. Differences in continuous variables were evaluated by the t test, using the log-transformed values for neutralizing antibody titers. All analyses were conducted using STATA (STATA statistical software release 9.0). Of the 55 anti-HPV31-positive samples in ELISA, 43 (78%) revealed evidence of neutralizing activity. The ELISA antibody titers ranged from 25 to 1,600, whereas the neutralizing titers ranged

from 25 to 6,400. The mean neutralizing titer was 496.5 for the ELISA-positive samples, 305.2 for sexually active women, and 822.1 for prostitutes ($P = 0.18$). The geometric mean titer of neutralizing antibodies was 112.2 for sexually active women and 208.3 for prostitutes ($P = 0.18$). It should be noted that the neutralizing titers were higher than the ELISA titers for 21 subjects, the same for 20 subjects, and lower for 3 subjects. Neutralizing antibodies (titer, 25) were detected in 2 of the 22 virgins investigated. These two women were also anti-HPV31 positive by ELISA. The fact that 2 of 22 women reporting as virgins had HPV31 antibodies detected by ELISA is in agreement with reports that HPV infection can occur following nonpenetrative sexual contact (24). The ELISA and neutralizing titers were low after natural infection (25 to 6,400), and the ELISA and neutralizing titers were slightly higher in prostitutes than in the other women. This could be related to a much higher rate of reinfection for prostitutes, which could boost the immune response. Our results indicate that 22% of anti-HPV31 ELISA-positive sera were not found to be neutralizing. This may indicate the existence of false-positive ELISA results, that ELISA is more sensitive than the neutralization assay in detecting anti-HPV antibodies, or that the neutralizing titers are lower than the ELISA titers (i.e., that some antibodies are not directed at neutralizing epitopes). This is in agreement with findings reported by Buck et al. (3) and Pastrana et al. (13) and could be related to differences in antibody-VLP avidity or the types of antibodies involved in the neutralization and ELISA tests. In addition, it is clear that individual HPV31 MAbs are not equal in neutralizing capacity, since several MAbs showed strong reactivity to HPV31 VLPs but only minimal binding to infectious pseudovirions, a finding recently reported for HPV16 by Culp et al. (6). They suggested that this could be related to the fact that surface epitopes on L1L2 pseudovirions produced in 293FT cells are closer to epitopes of authentic virions than are epitopes present on L1-only VLPs.

Investigation of the protective role of anti-HPV16 antibodies after natural infection indicated that ELISA titers of >400 to 800 at more than two consultations are associated with a reduced risk of subsequent infection (10). Assuming that anti-HPV16 and anti-HPV31 antibody titers are at similar levels after natural infection, this would suggest that fewer than 10% of anti-HPV31-positive subjects had protective levels of anti-

bodies. However, Viscidi et al. (22) showed in a large prospective study conducted in Costa Rica that seropositive women had the same risk of subsequent HPV infection as seronegative women, with the most likely explanation being that the low level of antibodies elicited after natural HPV infection is not protective against viral reinfection or reactivation.

We thank Richard Roden (John Hopkins Hospital, Baltimore, MD) for the kind gift of the anti-HPV18 L2 antibody, Rebecca Font (Institut Catala d'Oncologia) for statistical analysis, and the women interviewed and medical personnel at the Dermatology Clinic of Monte Naranco Hospital in Oviedo, Spain, and Costa Ponent, Hospitalet de Llobregat, Spain.

This research was supported by funding from the Institut National de la Santé et de la Recherche Médicale (INSERM) and by a special grant from the Association Vaincre la Mucoviscidose. Maxime Fleury was supported by a fellowship from the Ministère de l'Enseignement Supérieur et de la Recherche.

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