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Measurement of the Humoral Immune Response following an Incident Human Papillomavirus Type 16 or 18 Infection in Young Women by a Pseudovirion-Based Neutralizing Antibody Assay▽

Jane Steele,[1]*† Stuart Collins,[2]† Kaisheng Wen,[1] Gordon Ryan,[1] Christothea Constandinou-Williams,[1] and Ciaran B. J. Woodman[1]

CRUK Institute for Cancer Studies[1] and CRUK Clinical Trials Unit,[2] University of Birmingham, Edgbaston, Birmingham B15 2TT, United Kingdom

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We have evaluated a neutralizing antibody assay which uses human papillomavirus (HPV) type 16 (HPV-16) and HPV-18 pseudovirions carrying a secretory alkaline phosphatase reporter gene and which can potentially measure functionally relevant HPV type-specific neutralizing antibodies. The reproducibility of the assay was excellent; for HPV-16, the intra- and interassay kappa values were 0.95 and 0.90, respectively; and for HPV-18, the corresponding values were 0.90 and 0.90. This assay was used to describe the kinetics of the neutralizing antibody response in a cohort of 42 young women who were recruited soon after first intercourse and who first tested positive for HPV-16 DNA or HPV-18 DNA, or both, during follow-up. Most women seroconverted following the first detection of type-specific HPV DNA and remained seropositive until the end of follow-up. Our findings are broadly consistent with those of two other cohort studies which have measured the serological response following an incident infection by using the technically simpler virus-like-particle-based enzyme-linked immunosorbent assay.

Cervical human papillomavirus (HPV) infection is a common sexually transmitted disease, and infection with high-risk types is now considered a necessary cause of cervical cancer. The kinetics of the humoral immune response following an incident HPV infection in naïve women are not well defined. There are two major difficulties. First, because cervical infection with HPV is an asymptomatic event, its onset can never be precisely defined. However, it can be approximated by making frequent observations at short intervals in women who are unexposed at recruitment. These women should be recruited soon after they have first had sexual intercourse, because the longer the interval between first intercourse and study entry, the more likely it is that a woman will have acquired and then cleared at least one HPV infection during this time (3, 9). Only two studies have fulfilled these criteria (2, 5). Both recruited university students; one included 42 women with an incident HPV type 16 (HPV-16) infection and 30 with an incident HPV-18 infection; the other included 28 women with an incident HPV-16 infection. The second difficulty relates to the assay used to measure the serological response. Both of the studies cited used virus-like-particle (VLP)-based enzyme-linked immunosorbent assays (ELISAs). The integrity of the ELISA depends on the maintenance of intact VLPs; disrupted or incorrectly folded VLPs may lead to the detection of nonneutralizing and cross-genotype-reactive antibodies, thus complicating the interpretation of the results. Competitive radioimmunoassays (cRIAs), which specifically measure HPV type-specific neutralizing antibody in serum by using mouse monoclonal antibodies as competitors, have also been developed. The cRIA generally has lower backgrounds than the ELISA, is more sensitive, and is less likely to be influenced by impurities. However, like any serological assay based on competition, it fails to detect serum antibodies that fail to bind to or compete with the epitopes that bind to the competing antibody.

We have evaluated a neutralizing antibody assay, first described by Pastrana et al. (7), which uses HPV-16 and HPV-18 pseudovirions (PsVs) carrying a secretory alkaline phosphatase (SEAP) reporter gene and which can potentially measure functionally relevant HPV type-specific neutralizing antibodies. Having first defined the reproducibility of the assay, we describe the kinetics of the neutralizing antibody response in a cohort of 42 young women who were recruited soon after first intercourse and who first tested positive for HPV-16 DNA or HPV-18 DNA, or both, during follow-up.

MATERIALS AND METHODS

Study population. The samples used to validate this assay were collected during a prospective cohort study investigating the natural history of cervical neoplasia. The study design and the characteristics of the study population have been described elsewhere (9). In brief, 2,011 women aged 15 to 19 years were recruited from a single family planning clinic in Birmingham, United Kingdom, between 1988 and 1992 and were asked to return at intervals of 6 months; follow-up ended on 31 August 1997. At each follow-up visit, one cervical sample was taken for cytological examination, followed by the collection of another sample which was stored for subsequent virological examination. The women were also asked to provide a serum sample. All women with an abnormal smear, irrespective of its severity, were immediately referred to a colposcopy clinic for histological examination. Colposcopic and cytological surveillance was maintained in these women, and treatment was postponed until there was histological evidence of high-grade cervical intraepithelial neoplasia of grade 2 or 3, at which point the women left the study. After all clinical follow-up had ended, cervical cytology samples were tested for the presence of HPV DNA by PCR with a

* Corresponding author. Mailing address: CRUK Institute for Cancer Studies, University of Birmingham, Vincent Drive, Edgbaston, Birmingham B15 2TT, United Kingdom. Phone: 0121 414 7458. Fax: 0121 414 4486. E-mail: j.c.steele@bham.ac.uk.
† These authors made equal contributions.
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general primer pair (primers GP5+ and GP6+), and further PCR tests were done with type-specific primers with samples that were HPV positive after ethidium bromide staining (9). The study was approved by the appropriate research ethics committee, and informed verbal consent was obtained from all women.

The measurement of intra- and interassay variations used 42 serum samples taken from 21 women in this cohort: the corresponding cytological samples had been found by PCR with primers GP5+ and GP6+ to test positive for HPV-16 DNA or HPV-18 DNA, or both, either alone or in the presence of other HPV types, or to test negative for HPV DNA. We also tested a further seven serum samples which had been included in the WHO collaborative study on the standardization of the detection of antibodies to HPVs. These sera were kindly supplied by Morag Ferguson (Division of Virology, National Institute for Biological Standards and Control, South Mimms, United Kingdom) and had been taken from women who were described as having good histories of benign natural monovalent infections (three with HPV-16 infections, two with HPV-18 infections, and one with infections with both types) and a woman who was described as being negative for HPV.

Following the completion of assay validation, a preliminary analysis of the neutralizing antibody response associated with the first detection of HPV-16 or HPV-18 DNA was undertaken with a subset of the study population who had a normal smear, who were HPV DNA negative at the time of study entry, and who had further follow-up. This subset of 42 women included 12 women who tested positive for HPV-16 DNA in cytological material, 15 who tested positive for HPV-18 DNA, and 15 who tested positive for both types.

Preparation of PsVs. The preparation of PsVs for use in the neutralization assays was performed essentially as described by Pastrana et al. (7). Briefly, pseudovirus stocks were prepared by transfection of 293TT cells with vectors (all kindly provided by John Schiller, Division of Basic Sciences, National Cancer Institute, Bethesda, MD) expressing HPV-16 L1 (p16L1h), HPV-16 L2 (p16L2h), HPV-18 L1 (peL1fB), HPV-18 L2 (peL2bhb), and bovine papillomavirus type 1 (BPV-1) L1/L2 (pSheLL) together with SEAP (pYSEAP). For the production of HPV-16 and HPV-18 PsVs, L1-L2 pairs were cotransfected with the reporter plasmid. Three days following transfection, cells were harvested and pseudovirus was isolated over Optiprep gradients (Accurate Chemical, Westbury, NY). For each pseudovirus, fractions were titrated to determine the minimum amount of PsV required to give a robust signal in an assay for SEAP activity following infection of 293TT cells. This concentration of PsV was then used in subsequent neutralization assays. For the titration, 293TT cells (3×10^4/well) were plated in 96-well plates in neutralization assay medium (NAM; 100 μl/well) comprising Dulbecco modified Eagle medium without phenol red (Gibco-Invitrogen), 10% heat-inactivated fetal calf serum, 1% minimal essential medium nonessential amino acids (Gibco-Invitrogen), 1% Glutamax (Gibco-Invitrogen), and 1% antibiotic-antimycotic (Gibco-Invitrogen); and the plates were incubated for 2 to 5 h at 37°C. Serial dilutions of the PsVs (1:200 to 1:50,000) were prepared by using siliconized tips in 96-well polystyrene plates (Costar; Corning) in triplicate, with and without heparin (Sigma) treatment (incubation for 1 h at 4°C); added to the preplated cells (final volume, 200 μl/well); and incubated at 37°C for 72 h. The cell supernatants were then assayed for alkaline phosphatase activity by chemiluminescence.

Chemiluminescent detection of SEAP. The method used for the detection of SEAP in the 293TT cell supernatants was again essentially that described by Pastrana et al. (7), and a Great Escape SEAP detection kit (BD Bioscience) was used. Dilution buffer from the kit was added directly to the wells of a black Optiplate-96 assay plate (45 μl/well; Perkin-Elmer). The 96-well plates containing 293TT cells and PsVs were lightly shaken, and 50 μl of a homogeneous cell suspension from each well was transferred to the corresponding wells of a fresh plate and centrifuged at $800 \times g$ for 5 min. The clarified supernatant from each well (15 μl) was removed and added to wells containing dilution buffer on the Optiplate-96 assay plate, and the plate was covered with plastic and then incubated for 30 min at 65°C. After the plate was cooled on ice for 2 to 5 min, assay buffer was added (60 μl/well) and the plates were incubated at room temperature for 5 min. A chemiluminescent substrate was added (50 μl/well), and following a final incubation of 20 min at room temperature, the plates were read on a microplate reader set at a glow endpoint of 0.2 s/well for raw data.

Neutralization assay. Neutralization assays were performed by first diluting various concentrations of the test serum in NAM in polystyrene 96-well plates and incubating titrated HPV-16, HPV-18, and BPV-1 pseudovirus stocks (in triplicate) for 1 h on ice in a total volume of 100 μl. Triplicate wells of titrated pseudovirus alone were also set up to determine the maximum signal in the absence of neutralizing serum. The contents of each well were transferred to 96-well tissue culture plates that had previously been seeded with 293TT cells (3×10^4/well) in 100 μl NAM. The plates were incubated for 72 h at 37°C, and the SEAP activity in the cell supernatants was determined by the chemiluminescent assay described above. The 50% neutralizing titer, i.e., the highest dilution at which the signal was less than 50% of the mean signal seen in the control wells without serum, was determined for each serum sample. A serum sample was considered positive by the HPV-16 or the HPV-18 assay if it was neutralizing at a dilution at least fourfold higher than the titer observed in the BPV-1 assay.

Measures of assay reliability. Reliability was measured by using weighted kappa statistics. Neutralizing titers were measured on an ordinal categorical scale with categories of 0 (negative seroresponse), 40, 120, 360, 1,080, 3,240, and 9,720. Whereas the unweighted kappa statistic depends on exact agreement only, the weighted kappa statistic is a more appropriate measure of reliability when there are more than two categories and these categories are ordered; in these circumstances, a difference of one category between repeated measurements is less serious than a difference of two or more categories. The weighted kappa statistic incorporates the ordinal nature of the measurements by giving greater weight to repeated measurements with smaller discrepancies. Weights were calculated by assuming an equal "distance" between categories (linear weighting), so that exact agreement received a weight of 1.0 and maximum disagreement received a weight of 0. Kappa takes a value of 1 for complete agreement and values close to 0 for disagreement. Landis and Koch (6) give ranges for interpreting the strength of agreement based on kappa values: <0.00 is poor, 0.00 to 0.20 is slight, 0.21 to 0.40 is fair, 0.41 to 0.60 is moderate, 0.61 to 0.80 is substantial, and 0.81 to 1.00 is almost perfect. To measure within-assay reliability, samples were tested in duplicate on the same plate; to measure between-assay reliability, the samples on these plates were retested with the same plate configuration.

Measurement of time to seroconversion. To estimate the time between the first acquisition of HPV-16 or HPV-18 DNA and the time to seroconversion to that type, we used the Kaplan-Meier method and the approach adopted by Carter et al. (2). The time to seroconversion was measured from the date of the visit corresponding to the collection of the sample in which HPV-16 or HPV-18 DNA was first detected until the date of the visit corresponding to the collection of the sample with which it was found that the woman first seroconverted to that type, with censoring done on the date of the last available serum sample. Both analyses were limited to women who acquired HPV-16 or HPV-18 DNA during follow-up, who were seronegative at all visits prior to HPV DNA detection, who had serology results available after the first visit in which an HPV DNA-positive sample was obtained, and who were not missing serology data from the visit that corresponded to that when HPV DNA was first detected, unless the woman was seronegative on the subsequent visit.

RESULTS

Assay reliability. In the first of the two experiments used to determine the reliability of the HPV-16 assay, the kappa value was 0.95 and the observed agreement was 99%. In the second experiment, the kappa value was 0.89 and the observed agreement was 97%. In the first of the two experiments used to determine the reliability of the HPV-18 assay, the kappa value was 0.82 and the observed agreement was 93%. In the second experiment, the kappa value was 0.90 and the observed agreement was 96%. For both the HPV-16 and the HPV-18 assays, these values suggest almost perfect agreement according to the scale of Landis and Koch (6), and indeed, most discrepancies were only of 1 titer.

When the interassay reliabilities of the HPV-16 and the HPV-18 assays were measured, four kappa values were calculated, and these correspond to the four ways in which one of the two replicates could be selected from the first experiment and compared with one of the two replicates from the second experiment. For the HPV-16 assay, the kappa values ranged from 0.75 (substantial agreement) to 0.90 (almost perfect agreement); the observed agreement was consistently greater than 90%. For the HPV-18 assay, the kappa values ranged from 0.72 (substantial agreement) to 0.90 (almost perfect agreement); the observed agreement was consistently greater than 89%.

Serological results. Forty-two women were tested for neutralizing antibodies to HPV-16 and HPV-18. The median number of visits from which at least one serum sample was available was 5 (range, 1 to 14 visits). Thirty-four women provided at least two serum samples: the median interval between the times of collection of the first and last serum samples was 1,325 days (range, 185 to 3,192 days). For all of these women, the first serum sample available was that taken at the time of study entry. For 19 women, the last serum sample available was that taken at the last visit.

All women included in this analysis were sexually experienced to some extent. The median interval between the date of first intercourse and the date of collection of the first serum sample (median "coital age") was 523 days (range, 18 to 2,230 days); the median number of sexual partners prior to collection of the first serum sample was two (range, one to nine partners).

The first available sample was seronegative for HPV-16 for 36 women and seropositive for HPV-16 for 6 women. Seventeen HPV-16-seronegative women first tested positive for HPV-16 DNA at a follow-up visit; 16 of these women had one or more evaluable serological samples at or subsequent to this visit, and 10 seroconverted to positivity for HPV-16; 2 were first found to be seropositive at the same visit in which HPV-16 DNA was first detected, 4 were first found to be seropositive at a subsequent visit, and 4 were first found to be seropositive before HPV-16 DNA was first detected (182, 218, 287, and 766 days prior to the detection of HPV-16 DNA, respectively). Of those who first tested positive for HPV-16 DNA and who subsequently seroconverted during follow-up, one each had a maximum antibody titer of 40, 120, 360, or 9,720. Six women seroconverted without ever testing positive for HPV-16 DNA during the study period, either before or after seroconversion, including one woman who was seropositive at the time of study entry. Of the 21 women who were seropositive sometime during follow-up, 15 remained seropositive until the end of follow-up (median interval between the first and last seropositive sample, 859.5 days [range, 102 to 2,058 days]; median number of consecutive seropositive samples, 5.5 [range, 2 to 10 samples], excluding 2 women who were seropositive on only one occasion); 6 became seronegative, and 4 of these women had a further seropositive sample.

The first available sample was seronegative for HPV-18 for 36 women and seropositive for HPV-18 for 6 women. Twenty-two HPV-18-seronegative women first tested positive for HPV-18 DNA at a follow-up visit; 20 of these women had one or more evaluable serological samples at or subsequent to this visit, and 9 seroconverted to positivity for HPV-18; 2 were first found to be seropositive at the same visit in which HPV-18 DNA was first detected, 6 were first found to be seropositive at a subsequent visit, and 1 was first found to be seropositive before HPV-18 DNA was first detected (231 days prior to the detection of HPV-18 DNA). Of those who first tested positive for HPV-18 DNA and who subsequently seroconverted during follow-up, 2 had a maximum antibody titer of 40, 2 had a maximum titer of 120, and 2 had a maximum titer of 3,240. Two women seroconverted without ever having tested positive for HPV-18 DNA; one had a titer of 40, and one had a titer of 1,080. Of the 16 women who were seropositive sometime during follow-up, 8 remained seropositive until the end of follow-up (median interval between the first and last seropositive sample, 1,106 days [range, 203 to 2,836 days]; median number of consecutive seropositive samples, 6 [range, 2 to 12 samples], excluding 3 women who were seropositive on only one occasion); 8 became seronegative, and 2 of these 8 women had a further seropositive sample.

The first evaluable sample from no woman was seropositive for both HPV-16 and HPV-18. Nine women whose first available sample was seronegative for both HPV-16 and HPV-18 first tested positive for both HPV-16 and HPV-18 DNA during follow-up; four seroconverted to positivity for HPV-16, and two seroconverted to positivity for both HPV-16 and HPV-18.

DISCUSSION

We believe that we have successfully validated and adapted for our purposes the neutralization assay first developed by Pastrana et al. (7). The reproducibility of the HPV-16 assay was excellent. The HPV-18 assay was more variable, but almost all discrepancies between replicates were limited to one titer category. The greater variability of this assay almost certainly reflects the greater instability of the HPV-18 PsVs. However, unlike for VLP-based assays, the impact of PsV degradation on the test results is at least immediately apparent.

Our findings are broadly consistent with those of two other cohort studies which have measured the serological response following an incident infection by a VLP-based ELISA. In women with incident HPV-16 infections, the median time from the time of first detection of HPV-16 DNA to the time of seroconversion was 10.6 months, which is greater than that reported by Ho et al. (5) (8.3 months) but similar to that reported by Carter et al. (2) (11.8 months). The median time from the time of first detection of HPV-18 DNA to the time of seroconversion was 31.3 months, which is substantially greater than that reported by Carter et al. (2) (12.8 months), although the numbers of subjects in both series were small. Carter et al. (2) also reported that at 18 months after the first detection of HPV DNA, 59.1% of the women with an incident HPV-16 infection and 54.8% of those with an incident HPV-18 infection had seroconverted; the corresponding seroconversion rates for women in our cohort were 61.1% and 36.2%, respectively. Ho et al. (5) reported a 12-month cumulative seroconversion rate following an incident HPV-16 infection of 56.7%; the corresponding rate for women in our cohort was 51.4%. In all three studies, most women who seroconverted remained seropositive until the end of follow-up.

Although we used a potentially more discriminating neutralizing antibody assay, we found, as have others, that some women fail to seroconvert when a VLP-based ELISA is used. For example, among those who failed to seroconvert to positivity for HPV-16, one had five opportunities to do so and another had six, during which time they tested positive for HPV-16 by PCR with primers GP5+ and GP6+ on three and four occasions, respectively. We also found that seroconversion could be transient and that seropositivity could be intermittent, although this may merely reflect antibody levels which fluctuate around the threshold of detection. Although we recruited women soon after first intercourse, some already appeared to have cleared a primary infection at the time of study entry. For example, of 12 women who were seropositive for either HPV-16 or HPV-18 at the time of study entry and who

tested negative for HPV DNA at that time, 4 had been sexually active for less than a year. Of course, it is also possible that vulval or vaginal infections that are not detected in cervical samples may also generate an HPV immune response (8). Although our observations are not novel, they again illustrate how DNA testing or serology may fail to identify those sexually active women who have previously been exposed to HPV. This would be an important consideration were the admission of such women to a prophylactic immunization program dependent on adducing evidence of no previous exposure to vaccine types.

Neutralization assays such as the ones that we have described are reported to have a higher specificity than the VLP-based ELISA (1, 4). Direct comparisons of the two suggest that the HPV-16 neutralization assay is possibly more sensitive than the HPV-16 ELISA, whereas the analytical sensitivities of the HPV-18 neutralization assay and the HPV-18 ELISA are similar (7). However, the neutralization assay is complex, and as with all cell-based assays, although it is ultimately successful, reproducibility was not easily achieved. In light of our experience with its use in a nonautomated setting, we cannot support the claim that this is a high-throughput assay suitable for use in large-scale epidemiological inquiries.

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