Cervical secretory immunoglobulin A to human papillomavirus type 16 (HPV16) from HPV16-infected women inhibit HPV16 virus-like particles-induced hemagglutination of mouse red blood cells

Leticia Rocha-Zavaleta a,*, Tonatiuh Barrios a, Alejandro García-Carrancá a,b, Víctor Valdespino c, Fernando Cruz-Talonia d

a Department of Molecular Biology, Institute of Biomedical Research, National University of Mexico, Mexico City, Mexico
b Research Division, National Cancerology Institute, Mexico City, Mexico
c Oncology Hospital, National Medical Centre XXI Century, Mexico City, Mexico
d National Centre for Clinics of Dysplasias (CENACLID), General Hospital of Mexico, Mexico City, Mexico

Received 20 April 2001; accepted 23 April 2001
First published online 11 May 2001

Abstract

Secretory immunoglobulin A (sIgA) antibodies are the first line of defence at the genital mucosa, and are thought to hinder viral infections by binding to conformational epitopes on the viral capsid. To investigate if cervical sIgA binds to conformational epitopes of the Human papillomavirus type 16 (HPV16), cervical mucus samples from 109 HPV16-infected patients were examined in a HPV16 virus-like particles-induced hemagglutination inhibition assay. 48 (44.1%) patients were able to inhibit hemagglutination. Inhibition of hemagglutination was associated with the presence of sIgA ($P = 0.001$). In conclusion, naturally occurring cervical anti-HPV16 sIgA binds to and hinders conformational epitopes on the viral capsid, suggesting that these antibodies might have a neutralizing capacity. ß 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Human papillomavirus; Virus-like particle; Secretory immunoglobulin A; Humoral response

1. Introduction

Infection of the uterine cervix with high-risk human papillomavirus (HPV) types, mainly types 16 (HPV16) and 18 (HPV18), is a sexually transmitted disease (STD) strongly associated with the pathogenesis of cervical cancer. Progression of low-grade squamous intraepithelial lesions (LGSIL) to high-grade squamous intraepithelial lesions (HGSIL) and cervical cancer is an infrequent event, suggesting that the natural course of viral infection may be modulated by host mechanisms of defence. Current understanding of the immune reactions controlling HPV infections is limited. It has been demonstrated that a humoral response is developed against the viral capsid proteins, systemic IgG [1–4] and mucosal IgA [5–8] antibodies reactive to HPV16 capsid proteins are commonly found in infected women, but seroconversion does not occur in all HPV16-infected women [9].

HPV enters the body through the genital mucosa, where secretory immunoglobulin A (sIgA) is the dominant antibody isotype. sIgA is thought to be the first line of defence at mucosal surfaces, and it plays a major role in neutralizing viruses infecting these areas [10]. Therefore it might be expected that an sIgA-mediated response to capsid antigens would emerge as a reaction against HPV infections. To provide protection, antibodies must neutralize viral particles by targeting conformational epitopes on the capsid proteins. It is unknown whether antibodies found in cervical mucus of HPV16-infected patients have neutralizing properties, therefore their participation in the natural evolution of HPV-associated lesions, as well as in the occurrence of relapses (appearance of previously treated lesions) and reinfections (appearance of lesions in new locations) remains unclear. A number of methods are available for measuring the neutralizing capacity of antibodies to HPV [11–14]. However, not all of them are suit-
able for the study of large numbers of human samples. Inhibition of HPV virus-like particles (VLP)-induced hemagglutination of mouse red blood cells (RBCs) has been used as a surrogate assay to evaluate neutralizing responses [11], and provides a useful methodology for the study of human cervical mucus samples.

To provide insight into the capacity of naturally occurring anti-HPV16 sIgA antibodies to bind to and prevent HPV16-VLP conformational epitopes from interacting with cellular receptors, sIgA antibodies from cervical secretions of HPV16-infected women were analyzed in a VLP-induced RBCs hemagglutination inhibition assay. Association of hemagglutination inhibition capacity with the presence of sIgA to HPV16 was also established.

2. Materials and methods

2.1. Human samples

Patients attending the National Centre of Clinics of Displasias (CENA CLID) at the General Hospital of Mexico (Mexico City, Mexico) were studied. Informed consent was obtained from all patients included in this work. Human material was handled according to institutional experimentation and safety guidelines. Patients were referred for colposcopy because of an abnormal Papanicolaou test. In all cases colposcopy-mediated diagnosis was corroborated by histopathology. Only patients with a confirmed LG SIL, who were positive for the presence of HPV DNA by PCR, high-risk HPV by Hybrid Capture II, and for the presence of HPV-16 by PCR, were included in this study. Cervical mucus was collected by washing the uterine cervix with 1 ml of sterile phosphate-buffered saline (PBS). Cell debris were eliminated by centrifugation at 13 000 rpm for 5 min. Mucus samples were stored at −70°C until tested. Colposcopy-directed biopsies were obtained, placed in sterile PBS and processed the same day.

2.2. HPV DNA amplification by PCR

All reagents used were purchased from Gibco BRL (Rockville, MD, USA). Tissue samples were treated with Proteinase K as described elsewhere [15]. DNA was extracted with phenol–chloroform and precipitated with ethanol. HPV DNA was amplified using the general MY09 (5′-CGT CCM ARR GGA WAC TGA TC-3′) and MY11 (5′-GCM CAG GGW CAT AAY AAT GG-3′) primers [16], that amplify a conserved 450 bp fragment from the L1 gene. DNA samples were denatured by heating the reaction at 95°C for 30 s. Annealing of primers was performed at 45°C for 30 s and extension at 72°C for 60 s. The cycle was repeated 30 times. PCR products were electrophoresed in 2% agarose gels, stained with ethidium bromide and visualized in a UV transilluminator. Internal control to assure DNA integrity was performed by amplifying the β-globin gene using the PC03 and PC04 primers as described previously [17]. As a positive control DNA from SiHa cells, which contain one or two copies of HPV16, was run concurrently with each reaction.

2.3. HPV16 DNA amplification by PCR

Specific amplification of HPV16 was achieved by using the Pr3 (5′-GTC AAA AGC CAC TGT GTC CT-3′) and Pr4 (5′-CCA TCC ATT ACA TCC CGT AC-3′) primers [18], that amplify a 499 bp fragment covering the HPV16-E7 plus fragments of the E6 and E1 genes. Template DNA was denatured at 95°C for 30 s. Primers were annealed at 57°C for 60 s and then extended at 72°C for 60 s. The cycle was repeated 30 times, and the PCR products were analyzed as described above.

2.4. Hybrid capture II assay

The Digene HPV Test Hybrid Capture® II (Digene Corp., Beltsville, MD, USA) for the detection of high-risk HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68 was used. 20 μl (250–500 ng) of DNA extracted from cervical tissue were placed in a tube containing 30 μl of specimen transport medium. 25 μl of NaOH-based denaturation reagent was added to each sample, the tubes were vigorously mixed and incubated at 65°C for 45 min. Hybridization and hybrid detection were performed according to the manufacturer’s instructions. Carrier DNA and constructed HPV16 DNA were respectively used as negative and positive calibrators, and were run in triplicate with each test. An assay was considered valid only when the results from the negative and positive calibrators showed a coefficient of variation < or equal to 25%, and the positive calibrator: negative calibrator mean values ratio was > or equal to 2.0. The cut off value for positivity was calculated for each assay and was defined as the mean RLU (relative light units) value of the positive calibrator.

2.5. HPV16-VLP ELISA

Recombinant HPV16 VLP-expressing baculoviruses were kindly donated by Dr. John Schiller (National Institute of Health, Bethesda, MD, USA). The VLP extraction and purification procedure has previously been described [19]. ELISA plates (Maxisorp, Nalge Nunc Int. Co. Naperville, IL, USA) were coated with 500 ng/well of purified VLP diluted in PBS at 4°C overnight. A standard ELISA was performed, using 100 μl of undiluted cervical mucus. A rabbit-anti-human IgA-secretory component (Dako Co., CA, USA) was used as a secondary antibody, followed by an anti-rabbit IgG-alkaline phosphatase-conjugated antibody (Sigma Chemical Co., St. Louis, MO, USA). The cut off value for positivity was calculated on the basis of distribution of absorbances of 50 women
without cytological, histological or molecular evidence of HPV, and was defined as the mean absorbance +2 S.D. after exclusion of outliers (cut off = 0.492).

2.6. Hemagglutination inhibition assay

RBCs for this assay were obtained and processed as described elsewhere [20]. For the antibody-mediated hemagglutination inhibition experiment, cervical mucus were heat-inactivated at 56°C for 30 min. 50 μl of cervical mucus were incubated with 250 ng of VLP, diluted in 50 μl of 10% bovine serum albumin in PBS, for 1 h at room temperature with gentle rocking. The mixture was then incubated with 100 μl of RBC at 4°C for 3 h as previously described [20].

2.7. Statistical analysis

Data were arranged in the form of 2×2 contingency tables to be analyzed by the Fisher Exact test. A basic significance level of P = 0.05 was considered in all tests.

3. Results

A total of 369 patients with a diagnosis of LGSIL were analyzed for the presence of HPV DNA. 249 (67.4%) patients were positive for the presence of HPV by MY09/11 PCR. High-risk HPV DNA was detected in 238 (64.4%) by Hybrid Capture II. Specific PCR demonstrated the presence of HPV16 in 117 (31.7%) patients. 109 patients who were found to be positive by all methodologies were selected for immunological analysis (Fig. 1).

Presence of sIgA antibodies to HPV16 capsid antigens in cervical secretions of HPV16-infected patients was investigated by ELISA using HPV16 VLP. 59 (54.1%) patients were found to be positive by all methodologies were selected for immunological analysis (Fig. 1).

These results suggest that over a half of HPV16-infected women are able to generate a mucosal IgA response to the virus.

To investigate whether antibodies contained in the cervical mucus of HPV16-infected patients were able to interact with viral particles, a hemagglutination inhibition assay was performed. A total of 48 (44.0%) patients showed the presence of antibodies with the capacity of inhibiting HPV16 VLP-induced hemagglutination (Fig. 2). 35 (72.9%) of them showed the presence of sIgA antibodies in ELISA (Table 1). Interestingly 13 patients, whose level of anti-HPV16 VLP sIgA was below the calculated cut off point, were able to inhibit VLP-induced hemagglutination (Table 1). However statistical analysis demonstrated that inhibition of hemagglutination is associated with the presence of sIgA antibodies to HPV16 VLP (P = 0.001).

4. Discussion

In this work the presence of sIgA antibodies to HPV16 VLP in cervical mucus of HPV16-infected women, and their capacity to inhibit VLP-induced hemagglutination were investigated. Cervical sIgA antibodies to HPV16 VLP were detected in more than 50% of the population studied. Previous studies had already demonstrated the presence of sIgA antibodies to HPV16 capsid antigens in HPV16-infected women [5,7]. However they reported a lower proportion of patients with sIgA than that detected.

Table 1

<table>
<thead>
<tr>
<th>Presence of sIgA to HPV16</th>
<th>Samples that inhibited VLP-induced hemagglutination (n = 48)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>35 (73.9%)</td>
</tr>
<tr>
<td>Negative</td>
<td>13 (27.0%)</td>
</tr>
<tr>
<td>Association (Fisher Exact)</td>
<td>P = 0.001</td>
</tr>
</tbody>
</table>
in the present work. Prevalence of anti-HPV antibodies may be affected by a number of factors; for instance, it has been demonstrated that seroreactivity to HPV16 VLP varies between women from different countries, tending to be more elevated in populations with a higher incidence of cervical cancer [21,22]. The present work was performed in a country with a high incidence of cervical cancer, we explored a population composed of Hispanic and local Indian women from poor Mexican settings, while Hagensee and colleagues examined a predominantly Caucasian population of students from the University of Washington, and Wang and collaborators analyzed a group of Swedish women. Seropositivity may also be affected by the stage of HPV-associated cervical disease [4]. Hagensee and colleagues included women with diagnosis of atypical squamous cells of undetermined significance (ASCUS), LGSIL and HGSIL, on the other hand the work by Wang involved women whose diagnosis varied from slight atypia to invasive cancer. Unlike the latter our work included exclusively patients with a diagnosis of LGSIL.

It has been proposed that the appearance of antibodies after the detection of HPV16-related lesions, may lead to protection against new infections with the same HPV type [5]. In order to be protective these antibodies must target conformational neutralizing epitopes on the viral capsid proteins. Major neutralizing epitopes have been found on the HPV16-L1 coat protein [23]. Therefore antibodies to these epitopes might be involved in the development of protection against HPV16 infection. To investigate if mucosal antibodies target conformational epitopes on the HPV16-L1 capsid protein, they were tested in a hemagglutination inhibition assay. Antibodies from 44.0% of patients inhibited HPV16 VLP-induced hemagglutination, suggesting that they recognize conformational epitopes on the capsid proteins. Hemagglutination inhibition assay has been regarded as a surrogate method to evaluate the neutralization potential of antibodies [11]. As far as we are aware this is the first report of the use of such methodology for the study of human secretory antibodies to HPV16.

Naturally occurring human neutralizing antibodies against HPV have been poorly documented. Neutralizing antibodies against HPV11 have been found in sera from patients with condyloma acuminata [12,24] and cervical intraepithelial neoplasia [24], but mucosal neutralizing human antibodies to HPV16 have not been reported yet. Here we report that human sIgA antibodies can interact with and hinder HPV16 VLP binding to receptors on RBCs. Although inhibition of the hemagglutination is not an absolute indicator of neutralization, it provides an introductory insight into the capacities of naturally occurring antibodies to HPV16. Here we showed that sIgA antibodies, generated during a natural infection by HPV16, can interact with HPV16 VLP, this is important since there is evidence showing that VLP display the same neutralization-related conformational epitopes as natural virions [25]. Consequently one would expect these antibodies to react also with complete viruses.

HPV16 VLP are currently being tested as a human vaccine [26] aiming to prompt the generation of antibodies that will eventually react against the infectious virus. Our observation that human antibodies developed as a response to HPV16 infection, bind to and inhibit HPV16 VLP provides evidence that HPV16 VLP and HPV16 share conformational epitopes involved in the generation of a response in humans, and supports the idea that VLP may be a successful vaccine.

Interestingly, 13 patients with a level of sIgA lower than the cut off point were able to inhibit hemagglutination. This might be explained either by the presence of low levels of highly avid antibodies, or by the presence of different isotypes of immunoglobulins (mainly IgG) which were not explored in this work. It is unlikely that antibodies generated to other HPV types during past infections are responsible for a cross-inhibition, since there are data suggesting that seroreactivity to a given type of HPV is mainly type-specific [1,27], and also that type-specific capsid antigens correlate with virion neutralization [28] making cross-neutralization a very rare event [13].

Elucidating the potential of the natural human antibody response to HPV16 is indeed a relevant matter, from the present work we conclude that secretory antibodies are generated against the virus in a high proportion of infected women, and that they have the capacity of inhibiting HPV16 VLP-induced hemagglutination. To determine if these antibodies can effectively neutralize the virus more specific methodologies such as the pseudovirion neutralization assay may be applied, in addition a follow up study of patients positive for the presence of hemagglutination-inhibiting sIgA, whose lesion has been treated is being performed to investigate if antibodies will protect them from reinfections.

Acknowledgements

We thank Dr. John Schiller (National Institutes of Health, USA) for the donation of the HPV16 VLP-expressing baculovirus. Miriam C. Guido, BSc, for excellent technical assistance. Dr. Jorge Ortiz L., Dr. Guillermo Gomez G., Dr. Miguel A. Castillo E. from the CENACLID for assistance and help in obtaining human material. The present work was performed to investigate if antibodies will protect them from reinfections.

References


