Binding by Immunoglobulin to the HPV-16-derived Proteins L1 and E4 in Cervical Secretions of Women with HPV-related Cervical Disease

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ABSTRACT

Although DNA of the human papillomaviruses (HPV) can be identified in epithelium of a large proportion of patients with genital squamous lesions, relatively little is known about the extent of the local host immune response to this virus. We analyzed cervical secretions from patients undergoing evaluation because of abnormal Papanicolaou smears (cervical biopsy showed nonspecific atypia, flat condyloma, or intraepithelial neoplasia), as well as controls, for immunoglobulin binding to proteins produced in vitro to HPV-16 L1, E4, and E7 open reading frames. Segments of the HPV-16 genome, including portions of the L1 (nucleotides 6153–6794), E4 (nucleotides 3399–3648), and E7 (nucleotides 686–880) open reading frames, were cloned into pATH vectors and expressed as tryptophan synthetase E fusion proteins in Escherichia coli and used as a source of study antigens. Fusion proteins containing the HPV L1, E4, and E7 polypeptides were found to be distinct by molecular weight (59,000; 45,000; and 42,000) as well as by immunological determinants recognized by heterologous immune sera. Of 8 cervical intraepithelial neoplasia lesions tested by RNA-RNA in situ hybridization, 7 were found to be positive for HPV-16-related nuclear acids, in contrast to none (0 of 4) in the condyloma group (three positive for HPV DNA other than type 16). Immunoglobulin in cervical secretions showed reactivity to HPV type 16 E4 or L1 or both, with highest binding in patients with cervical intraepithelial neoplasia (P < 0.01 for HPV-16 L1 and E4 compared with controls). Binding was not tryptophan synthetase E dependent and was, in general, coincident for the HPV-16 E4 and L1 proteins. We conclude that study of cervical secretions, using a quantitative assay for immunoglobulin binding to HPV-16 proteins produced in vitro, may be useful to document the quality and quantity of the immune response of the host to this important human pathogen.

INTRODUCTION

HPVs are a heterogeneous group of double-stranded DNA viruses which can infect squamous epithelium of the genital tract and are associated with genital warts, precancers (intraepithelial neoplasms), and invasive carcinomas (1–6). Although HPV-16 is the principal type in intraepithelial neoplasms of the cervix, and HPV nucleic acids have been detected in a large proportion of genital squamous lesions, relatively little is known about the cell biology of host infection by these viruses, specifically which HPV-encoded proteins are produced with infection and the extent of the host immune response to these viral proteins. These questions have recently been addressed using techniques of fusion protein technology, in which DNA sequences information from previously characterized HPVs has been used to produce plasmid constructs, in which a defined HPV ORF can be expressed as a fusion protein and used to generate antisera or provide a target for analyzing the host response. Using this approach, investigators have identified a humoral immune response to HPV-6 and -16 proteins in study populations (7, 8), and type-specific epitopes have been defined for HPV-6b which react with human sera (9). These studies indicate that it may be possible to identify specific determinants on macromolecules encoded by HPV DNA and which elicit the host immune response.

Because “genital" HPV DNA types may be recovered in the conjunctiva, oropharynx, larynx, and subungual regions (10–13), the relevance of a humoral response in serum to genital infection remains unclear. One approach to this problem centers on the local immune response in the cervical mucosa. Antibodies reacting to herpesvirus, chlamydial, and gonococcal antigens, principally of secretory IgA isotype, have been isolated from this region (14–17). In a recent report, detection of IgA antibodies reacting to bovine papillomavirus virion proteins in cervical mucous specimens of a proportion of women with abnormal Papanicolaou smears was described (18). In this preliminary study, we analyzed local cervical immune reactivity to fusion proteins produced in vitro to portions of the HPV-16 L1, E4, and E7 ORFs. We selected these ORFs because we and others have been able to demonstrate that HPV-16 L1 and E4 are expressed in some cervical precancers and E7 expression has been documented in cell lines from cervical cancers (19–22). We report the presence of antibody-binding activity to the L1 and E4 (but not E7) proteins in a subset of women with cervical precancer lesions. In the process of developing a quantitative assay for binding activity, we have purified each study protein (L1, E4, E7) through preparative electrophoresis and elicited specific hyperimmune sera to them. The relationships among HPV-specific binding activity in cervical secretions, lesion type, and associated HPV nucleic acids are discussed.

MATERIALS AND METHODS

Preparation and Characterization of Study Proteins

Expression and Purification. Segments of the HPV-16 genome including portions of the L1, E4, and E7 open reading frames were cloned into GEM Vectors (Promega, Madison, WI), partially sequenced, and inserted in the appropriate reading frame in selected pATH vectors (23). The L1, E4, and E7 peptides corresponded to nucleotides 6153–6794, 3399–3648, and 686–880 of the HPV-16 genome (Fig. 1) (24), representing 54, 76, and 65% of these ORFs, respectively. Using a protocol modified from Firzlaff et al. (7), we used constructs to transform into Escherichia coli MM294, and transformants containing the insert sequences were incubated overnight with shaking in minimal media with tryptophan (7). They were then diluted 1/10 in minimal media without tryptophan, shaken for 2 h, and then induced with indoleacrylic acid. Samples were shaken at 32°C for 2 h, pelleted, and resuspended in lysis buffer. Whole lysates were resolved on SDS-poly-
acrylamide gel electrophoresis, and the size of the fusion proteins was recorded.

Fusion proteins were purified by preparative polyacrylamide gel electrophoresis. A 5.9–12.8 mg of each sonicated preparation was electrophoresed on a 10% SDS polyacrylamide slab gel, pH 8.8 (200 V, 22°C, about 4 h) in an LKB model 2001 apparatus as described previously (25), and electroelution was accomplished by a modification of the method described by Hunkapiller et al. (26). The purity of each gel-eluted band was assessed by reelectrophoresis on an analytical 10% SDS polyacrylamide gel, and immuno-reactivity as well as antigenic specificity studied by immunoblotting, as described below. To determine whether the bacteria-coded portion of the fusion protein contained constituents also recognized by cervical mucous specimens, 14 mg of noninsert containing pATH vector was fractionated and characterized by the same methods. Protein was measured by the procedure of Lowry et al. (27) using 2–50 μg of twice-recrystallized bovine serum albumin (fraction V; Eastman Kodak Co., Rochester, NY) as the standard.

Analytic Polyacrylamide Gel Electrophoresis. Whole cell lysates and their electroeluted fractions were examined under SDS-denaturing conditions. A 10% system as described by Laemmli (28) was used. Aliquots of 1 to 5 μg protein were dissolved in 0.5 μl Tris buffer, pH 6.8–5% glycerol–0.001% bromophenol blue. Subsequently, the gels were fixed in 50% methanol–10% acetic acid (30 min) followed by 10% glacial acetic acid (30 min). Protein on gels was detected using a silver stain technique as described by Merrill et al. (29). Molecular weight standards used were: phosphorylase B, 97,400; bovine serum albumin, 66,000; ovalbumin, 44,000, and carbonic anhydrase, 29,000.

Immunological Methods

Elicitation of Specific Antisera. Antibodies to the fusion proteins were obtained through s.c. injection of 50–150 μg of antigen, dissolved in saline and emulsified in an equal volume of Freund’s complete adjuvant (Difco Laboratories, Detroit, Michigan), into the hind footpads of New Zealand white rabbits. A panel of sera at peak titer was subsequently selected for study by a quantitative ELISA described below. Sera were studied at 1:50,000–1:100,000 dilution, with and without prior absorption with bacterial lysates as indicated. Individual antigenic specificities were identified by immunoblot (see below).

Immunoblot Technique. This was carried out by a modification of the method of Gershoni and Palade (31). Incubation for 2 h at 25°C with first antibody (heterologous immune serum or preimmune serum, diluted 1:50,000) was followed by exposure to a 1:600 dilution of alkaline phosphatase-conjugated anti-rabbit IgG and L chain (Kirkegaard and Perry, Gaithersburg, MD) for 1 h at 25°C and then substrate (5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium, both from Sigma Chemical Co.). To determine the antigenic specificity of antisera, the fusion protein preparation or control proteins (sonicated pATH vector after induction or ovalbumin) were added in 0–through 50-μl amounts to antiserum (0.003 ml), incubated for 14 h, and pelleted (5000 rpm, 15 min). Supernatant (with and without absorption) was then tested for residual binding to the study antigens impregnated onto nitrocellulose, using alkaline phosphatase-conjugated second antibody in the standard immunoblot technique above.

Histochemical Verification of Study Antisera. To verify that the study antigens elicited antisera which cross-reacted with HPV-related proteins, serial sections from biopsied lesions containing HPV-16 nucleic acids were analyzed by histochemistry, as previously described (21). Sections were incubated with anti-L1, E4, and -E7 sera at 1:800 dilution in 1% goat serum, and reaction products were detected with a biotinylated secondary antibody and avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA), using 3,3′-diaminobenzidine (0.5 mg/ml; Sigma) as substrate. Controls included sections exposed to antisera but preincubated with their respective antigen.

ELISA. The L1, E4, E7 fusion proteins or control protein, tryptE from the non-HPV gene insert-containing pATH vector, were adsorbed to the bottom of a 96-well microtiter plate (Falcon 3912; Becton Dickinson, Oxnard, CA) by incubating 100 μl of coupling buffer/well containing 0.2 μg of antigen. After 3 h at 37°C, the plates were washed and blocked with buffer containing 0.5% casein (Sigma) (12 h, 4°C). Study cervical mucous specimens were applied at a 1:25 and a 1:100 dilution. After extensive washing, followed by incubation with alkaline phosphatase-conjugated goat anti-human immunoglobulin (Kirkegaard and Perry), substrate (number 104 phosphatase tablets; Sigma) was added and the plate read at 2, 5, and 20 h on a Titertek Multiskan apparatus (Flow Laboratories, McLean, VA). A designation of positive was assigned only when net absorbance at 405 nm of triplicate specimens was >0.140, this finding could be repeated with a second set of triplicate specimens, and reactivity directed against HPV-16-coded protein made up at least 35% of the read-out (absorbance) value.

Source and Analysis of Clinical Material

Patient and Sample Selection. Women presenting to the University of Virginia Colposcopy Clinic for the evaluation of an abnormal Papainoclaou smear were selected as patient cases. A speculum was inserted, and cervical mucus was aspirated from the cervical os and placed in normal saline with 1% bovine serum albumin. Samples were then centrifuged and the supernatants were sonicated and stored with 0.01% sodium azide at 4°C. As controls, cervical mucus samples from women presenting for routine gynecological care, without an abnormal Papanicolaou smear or a cervical lesion and age-matched with patient cases (above), were identically processed and tested.

Morphological Analysis of Lesions. Biopsies obtained at the time of colposcopic examination were fixed in formalin, sectioned, and classified according to previously defined criteria (32) into the following categories: (a) negative or nonspecific atypia, (b) flat condyloma, and (c) CIN. The last two categories correspond approximately to mild-moderate and severe dysplasia/carcinoma in situ, respectively. The last category (CIN) has been closely associated with HPV type 16 nuclear acid sequences in previous reports (3, 32).

Analysis of Specimens for HPV DNA. In order to determine the type of HPV nucleic acids associated with the lesions under study, RNA–RNA in situ hybridization of biopsy material was performed using 35S-labeled RNA probes obtained from HPV type 16, as previously described (33). As a control, serial sections were incubated with a mixed probe consisting of HPV-11 and HPV-18 nucleic acids. The resulting hybridization signals were interpreted as either positive or negative for HPV-16 nucleic acids or HPV nucleic acids of types other than type 16 (i.e., HPV-11 or -18).

Statistical Analysis. Since obtained values displayed a nonparametric distribution, a Wilcoxon rank sum test (34) was used to compare immunoglobulin binding among the patient groups. The primary data were analyzed in two ways: (a) as net absorbance at 405 nm, obtained by subtraction of tryptophan E binding from binding to the fusion protein and (b) as a ratio, Abs fusion protein/Abs tryptophan E, where net absorbance favoring the fusion protein would have a ratio of >1.00. Only in the case in which both analyses (e.g., net absorption and ratio)
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Fig. 2. SDS polyacrylamide gel electrophoresis of bacterial lysates, including those containing fusion proteins L1, E4, E7, used in this study. After electrophoresis of noninduced (Un-I) and induced (I) lysates from pATH vectors containing HPV-16 nucleotide sequences 6153-6794, 3999-3648, or 668-880, the gels were stained as described by Merrill et al. (29). Subsequently, induced components were identified (arrows), cut from the gel, and electroeluted according to the method of Hankapiller et al. (26). Tryptophan synthetase E was similarly prepared from unmodified pATH vector. Molecular weight markers were (in thousands) carbonic anhydrase (29), ovalbumin (45), bovine serum albumin (66), and phosphorylase b (97.4).

Fig. 3. Specificity for an HPV-16-coded protein of immune sera as demonstrated by immunoblotting. Twenty μg of protein L1-containing pATH lysate (lane L1), in parallel with a control pATH lysate containing tryptophan synthetase E (lane P), was electrophoresed and then transblotted onto nitrocellulose prior to incubation with a 1:100,000 dilution of immune serum A, elicited to a fusion protein encoded by HPV-16 nucleotides 6153-6794. Preexposure of the same serum to progressively increasing amounts of L1 protein-containing pATH lysate diminished reactivity to the L1 band (first lane of panels 2 and 3) and less so to the tryptophan synthetase E band (second lane of panels 2 and 3). Exposure to similar amounts of ovalbumin did not diminish reactivity to the L1 or the tryptophan synthetase E proteins (panels 4 and 5).

of group data demonstrated \( P < 0.05 \) was a statistically significant result designated.

RESULTS

Partial Purification of Fusion Proteins

Bands representing the induced fusion proteins were readily identified (Fig. 2), cut from the gel, and electroeluted. Purified from lysates was 0.4 mg of L1 fusion protein, 0.35 mg of E4 fusion protein, and 0.32 mg of E7 fusion protein, for yields of 6.7, 2.9 and 2.5%, respectively. By their migratory rate through polyacrylamide, fusion proteins containing L1, E4, or E7 (Fig. 2, components marked by arrows in lanes 2, 4 and 6) could be distinguished from the inducible tryptophan synthetase E in noninsert-containing pATH (lane 8). Furthermore, from their physicochemical properties, fusion proteins in lysates from pATH with unique gene inserts were quite distinct electrophoretically. Compared with coincidentally run standards, bands corresponding to the L1, E4, and E7 fusion proteins demonstrated unique molecular weights (59,000, 45,000, and 42,000, respectively).

Immunological Analysis of Study Antigens

Characterization with Heterologous Immunoglobulin. Reactivity of hyperimmune sera, used below for analysis of determinants on study antigens, was first defined by immunoblot analysis. Insert-containing pATH lysate electrophoretically separated on an SDS-denaturing polyacrylamide gel was used, and
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Evidence for Unique Determinants. We next investigated whether immunologically unique viral determinants were present on fusion proteins isolated from the L1, E4, and E7 systems. To determine this by a methodology which was quantitative, the fusion proteins isolated by electrophoretion after electrophoretic separation were examined for binding with specifically immune sera by an enzyme-linked immunosorbent assay (Fig. 4). While binding of serum A elicited to HPV-16 L1 was strongest with the homologous macromolecule (e.g., L1) with an end point (A405) >2.5-fold that of tryptophan synthetase E, it demonstrated minimal binding to E4 constituents (Fig. 4). Evidence for unique determinants was found also with serum B elicited to E4: only a small amount of binding was detected to L1 (Fig. 4). This together with the immunoblot absorption experiments (above) indicate the presence of unique, nonbacterial components on the L1 and E4 fusion proteins. Similarly, serum A could not be blocked by preincubation with control pATH vector lysate (up to 50 µg) but was markedly inhibited with relatively small quantities (5 µg) of L1-containing pATH lysate (Fig. 5, bottom). Immune sera elicited to E4 reacted similarly, being readily inhibited only by E4-containing pATH lysate (Fig. 5, top).

Histochemical Localization of Study Antisera. L1-specific serum produced nuclear staining in the superficial cells of a variety of genital lesions, paralleling the site of HPV viral replication (Fig. 6B) and assembly (Fig. 6C). Anti-E4 antisera produced cytoplasmic staining in the same cell population as L1 (Fig. 6D), as previously described (21). Anti-E7 antisera, elicited to a construct representing 65% of the E7 ORF, failed to produce specific immunoreactivity in biopsy material (data not shown), despite previous reports identifying this protein by Western blot in cancers and their derived cell lines.

Presence of Viral (HPV-16) DNA. Analysis of Cone biopsy material performed on patient cases revealed that 0% (0 of 3), 0% (0 of 4), and 87% (7 of 8) of NSE, condyloma, and CIN, respectively, were positive for HPV-16 nucleic acids by RNA-RNA in situ hybridization. Hybridization signals were observed,
using the $^{35}$S-labeled HPV-16-derived RNA probe, which were confined principally to the superficial epithelium (similar to that depicted in Fig. 6B). In the condyloma group, 15% (3 of 4) tested positive for HPV DNA other than type 16.

**HPV-16-specific Binding by Immunoglobulin in Cervical Secretions.** To determine by a quantitative technique whether immunoglobulin in cervical secretions was reactive with the HPV-16-coded proteins L1, E4, and E7, an ELISA technique, standardized for this antigen system (described above), was used to compare binding for each fusion protein, purified by electroelution. Cervical secretions showed reactivity to HPV-16 E4 or L1 or both, with highest binding recorded in patients with CIN (Fig. 7). Differences were statistically significant for the HPV-16 L1 and E4 antigens, when the control and CIN groups were compared ($P < 0.01$). A similar significant difference was found for the HPV-16 L1 antigen, comparing the CIN and the NSE groups ($P < 0.05$). Binding to the bacterial (vector-coded) portion of the fusion protein did not account for these findings, since reactivity to tryptophan synthetase E (comprising the bacterial component) was simultaneously determined, and its value subtracted in determining HPV-16 protein-specific binding. Nonspecific binding was unlikely, given minimal reactivity of cervical mucous specimens with control protein, $\beta$-lactoglobulin (data not shown) and with HPV-16 E7 (Fig. 7). In general, binding for E4 and L1 was coincident in the same cervical specimen.

**DISCUSSION**

This study was designed to determine whether cervical secretions from women with HPV-associated genital precancers contained antibodies reactive with proteins associated with...
HPV type 16. The fusion proteins selected for study, e.g., those coded by a portion of the HPV-16 L1, E4, and E7 open reading frames, provided the opportunity to investigate reactivity against components of the major capsid protein (L1) of HPV-16, sharing considerable homology with other HPV types, a "late" protein (E4) with relatively little cross-reactivity to non-type 16 HPV by immunohistochemistry, and a potential onco-protein (E7). Antiserum to HPV-16 L1 will cross-react with capsid proteins of a variety of HPV types (22). However, in population studies, serum responses to this region have been rarely described (8, 9, 35). We have recently localized the HPV-16 E4 protein in tissue in a cytoplasmic distribution but have observed no cross-reactivity with other HPV types as of yet (21). Serological responses to this protein in a wide variety of subjects have been described, albeit infrequently (8). Localization of E7 by histochemistry has been unsuccessful, although this protein has been detected by Western blot in extracts from cervical cancers and cell lines (19–20). Serological reactivity to this protein has been demonstrated as well (35).

The finding of local (genital) antibody reactivity to HPV-16 L1 and E4 determinants is intriguing and suggests that, in patients with cervical precancers, immunoglobulins are generated locally in response to these infections and their related lesions. This observation would seem confirmed in a new series of cervical intraepithelial neoplasia patients just entering a longitudinal study, in which impressive reactivity (A_{00} > 0.50) to HPV-16 L1 was found in five additional patients and intermediate reactivity (A_{09} = 0.2–0.5) to both HPV-16 L1 and E4 was found in three more cases. One limitation of the current approach centers on the volume of cervical secretions available for study. In this series, volumes obtainable from individual patients permitted analysis by a quantitative and sensitive immunoassay but were insufficient to allow Western (immuno) blot analysis. Nevertheless, standardization of study protein concentrations (by Lowry assay), use of purified, characterized fusion proteins as antigens, and monitoring of reactivity against two control antigens (β-lactoglobulin, tryptophan synthetase E) were used to maximize the possibility that reactivity detected was due to specific binding of the HPV-16-derived proteins by immunoglobulin present in the cervical mucus samples. Additionally, the timing of sample procurement with the menstrual cycle could not be addressed. Studies indicate that the amount of mucosal antibody produced (and the amount of cervical secretions available for analysis) will vary with the menstrual cycle, being at its maximum just prior to ovulation (36). Hence, the proportion of positive samples may be underestimated if secretions are collected at random points in the cycle, as it was in the current study. It is possible that a bacterial protein copurified with a study fusion protein accounted for the reactivity which we detected. This possibility is less likely because (a) control patients in our study did not react with any of the fusion proteins (an unlikely event should a common bacterial component have copurified) and (b) cervical secretions from patients with cervical intraepithelial neoplasia did not react with HPV E7, even though the same pATH1 vector (the only likely source of a contaminating bacterial peptide) used to construct the HPV-16 L1 and E4 fusion proteins was used to construct E7.

The finding of specific antibody reactivity, particularly to HPV-16 L1 and E4 determinants on fusion proteins, is of interest in light of recent studies of serum reactivity to these antigens in women attending sexually transmitted disease and colposcopy clinics. Jenison et al. (9) isolated an epitope in the carboxy terminal region of HPV-6b which reacted with human sera and may be relatively specific for either HPV-6 or HPV-11. Although rabbit antisera directed to HPV-16 L1 proteins reacted to the HPV-6-derived constructs, a wide variety of human sera from patients with warts or abnormal Papanicolaou smears did not react to the HPV-16 L1 proteins. These data were interpreted as evidence either that HPV-16 L1 did not generate a serological immune response during HPV-16 infection in vivo or that the constructs used were unable to identify an existing immune response to an L1 epitope (9).

In this study, several samples of cervical secretions reacted to the fusion protein containing the carboxyl region of the HPV-16 L1 protein construct. Although the finding of immunity to this region of HPV-16 is unusual, the study of populations with HPV-related precancerous lesions would likely increase the opportunity to identify immunoreactivity to this open reading frame. Moreover, Dillner et al. (37) recently produced evidence that sera from patients with cervical neoplasms preferentially react to linear epitopes from the HPV-16 L1 region vis-à-vis control populations. Their results are consistent with the observations in this study and the hypothesis that immunoreactivity to HPV-16 L1 occurs preferentially in individuals with a genital lesion (37). Furthermore, in a new series of patients with cervical intraepithelial neoplasia yet to be reported, we found generous amounts of IgA by a radial immunodiffusion technique (Turbo RID; Binding Site Inc., San Diego, CA), being 410 μg/ml among 11 case samples with HPV-16 L1 reactivity and 420 μg/ml among 13 samples with

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4 S. A. Jenison, personal communication.
HPV-16 E4 reactivity in their cervical mucus. The concentration of immunoglobulins in cervical secretions was markedly decreased (<150 µg/ml) at days 12–14 of the menstrual cycle, as previously suggested by Wira et al. (36). Thus, although the data in this report suggest that this association extends to the mucosal region, definitive evidence regarding this issue will require comparative analysis of simultaneous serum and cervical samples from the same patient and use of a marker for the mucosal origin of immunoglobulin measured (e.g., secretory component). Such a study would confirm the current observations concerning HPV-16-related local immune reactivity and determine whether there are fundamental differences in either the source of reactive immunoglobulin or the HPV epitopes recognized between serum and mucosal sites.

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