Human Papillomavirus 8-L1 Immune Serum: A New Diagnostic Possibility for Epidermodysplasia Verruciformis-Specific HPVs

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Human papillomavirus (HPV) 8 induces flat macular skin lesions with a high risk of malignant conversion. A 600-bp fragment from the center of the major structural protein gene L1 was cloned into pEX2 to produce a β-galactosidase-L1 fusion protein. The expressed part of L1 is masked in intact virions and not detected by sera of infected patients. Immunization of guinea pigs with purified fusion protein raised high-titer antisera, which reacted with capsid proteins of HPV8 and closely related viruses in Western blot and indirect immunofluorescence tests. Structural proteins of HPV1, 2, and 3 were not detected, indicating specificity for the subgroup of ev-specific HPVs. The sera present themselves as convenient diagnostic reagents for the demonstration of infections with potentially oncogenic HPVs using routine immunofluorescence procedures. J Invest Dermatol 91:76—81, 1988.

A large group of rather closely related human papillomaviruses (HPV) infects patients with Epidermodysplasia verruciformis (ev) and induces flat warts and pigmented or achronic macules and papules, which tend to spread all over the skin [1,2]. Clinically apparent infections with these viruses are largely restricted to ev patients and depend on a genetic predisposition of the host. An impaired cell-mediated immunity is observed in most patients [3] and is assumed to increase the susceptibility to ev-associated HPVs. This is in line with finding these viruses in immunosuppressed renal allograft recipients [4—6], in an immunosuppressed patient with systemic lupus erythematosus [7], and in a patient with Hodgkin's disease [8].

The HPV infections in question are of considerable medical interest because they give rise to skin cancers in at least 30% of the patients. Malignant conversion was noted in up to 80% of Japanese ev patients [9], which may be due to the prevalence of HPV types with an increased oncogenic potential or to the influence of not yet characterized cocarcinogens. HPV5 and 8 predominate in malignant tumors and HPV14, 17, and 20 were detected once each [2,10].

Early detection of these infections is therefore particularly important in immunosuppressed patients to initiate regular, careful dermatologic examination.

A diagnosis may be provided by hybridizing biopsy DNA with cloned HPV reference DNAs, but a serologic detection of viral antigen would be obviously more convenient for the routine laboratory. It is hard to obtain high-titer monospecific antisera from ev patients, however, and type-specific antigens cannot be isolated for immunization of animals in view of frequently mixed infections with HPV3 or 10 and a possible additional infection with HPV2. We therefore tried to express the major capsid protein of HPV8 in bacteria to produce specific antigen in sufficient amounts for antisera induction. The antibody specificity was analyzed in Western blots and indirect immunofluorescence tests.

MATERIALS AND METHODS

Bacterial Strain and Plasmid The expression vector pEX2 has been described by Stanley and Luzio [11]. The HPV8 DNA was prepared from the 1655 bp BamHI-EcoRI fragment cloned in pBR322 [12]. For construction of pEX2/L1, the 600 bp HaIII fragment of HPV8 was ligated into the Smal site of pEX2 with T4-DNA-ligase. The ligated DNA preparation was used to transform competent E. coli POP2136 cells (kindly provided by O. Raimüer, Paris), which harbor a temperature sensitive cl-repressor gene of phage Lambda. The transformants were selected at 30°C in LB-broth supplemented with 100 μg/ml ampicillin. HPV8-L1-containing clones were identified by colony hybridization as described [13]. Plasmid DNA was prepared from recombinants [14] and analyzed by restriction enzyme digestion.

Expression and Purification of the HPV8-L1/β-Galactosidase Fusion Protein A 200 ml culture of a pEX2/L1 containing clone was grown with vigorous agitation (28°C) until it reached early/mid log phase (i.e., OD550 = 0.3 — 0.5; 1—2 x 10⁸ cells/ml). The incubation temperature was then raised to 42°C and induction of hybrid protein expression was carried out for 90 min before the bacteria were pelleted and resuspended in 1.5 ml Laemmli's sample buffer, boiled for 5 min, and analyzed by 8% SDS-PAGE [15]. After staining the electrophoresed gel with

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Coomassie brilliant blue, the acrylamide stripe with the 139 kD fusion protein band was cut out, frozen in liquid nitrogen, and pulverized. The fusion protein was extracted, lyophilized, and redisolved in phosphate buffered saline (PBS) to give an overall yield in the range of 5 mg.

Preparation of Antibody Against L1-β-gal Fusion Protein
Guinea pigs were immunized s.c. using about 20 mg of purified fusion protein with 0.75 ml of Freund’s complete adjuvant. The injections were repeated after 14 and 28 d, respectively, using the same amount of protein in Freund’s incomplete adjuvant; the injection at 28 d being given ip. The guinea pigs were bled 14 d after the third immunization.

Protein Extracts of Wart-Biopsies and Western Blotting
Individual HPV1- and HPV8-induced warts were minced up as finely as possible in 1–2 ml of 50 mM Tris, 5 mM EDTA, 150 mM NaCl, 0.5% NP40 using sea sand and a hand-held homogenizer. After sonification SDS was added to the mixture to a final concentration of 0.5%. The suspension was boiled for 5 min and insoluble material pelleted at 10,000 rpm for 10 min. The supernatant was dialyzed against three changes of water for 1 h each.

Two-microgram protein aliquots from human wart extracts were mixed with equal volumes of 2 × Laemmli buffer, boiled for 2 min, and separated on 12.5% polyacrylamide gels. The proteins were then transferred by Western blotting to nitrocellulose (Schleicher and Schüll) as described by Burnette [16]. The filters were coated by incubation in NET-buffer (0.15 M NaCl; 5 mM EDTA; 50 mM Tris, pH 7.4; 0.25% gelatine, 0.05% NP40; 2% BSA) for 30 min. The antibody reaction (antiserum were diluted 1:200 in NET buffer) was carried out for 3 h followed by three washes with PBS/0.1% Tween 20. Peroxidase linked anti-guinea-pig IgG as second antibody was diluted 1:1000 in NET buffer and incubated for 2 h followed by the washing procedure. Peroxidase binding was detected using chloro-naphthol and 0.025% H2O2.

Isolation of Virus Particles and Immune Electron Microscopy
Purification procedures for human papilloma viruses have been described [17]. Five microliters of virus suspension were mixed with 5 μl serum diluted in phosphate buffered saline. The assay was incubated overnight at 4°C and prepared for the electron microscope by negative staining [18].

Elimination of Anti-β-gal Antibodies
Anti-β-gal antibodies in guinea pig antisera and patient sera were absorbed with a lysate of an induced E. coli POP2136 pEX2 culture. Bacteria from 30 ml culture were pelleted and incubated for 90 min on ice in 175 μl 10 mM Tris HCl, pH 8.0; 10% sucrose with 1 mg/ml lysozyme. SDS was added to 2.5%, and the lysate was boiled for 5 min and passed through an 18 gauge needle three times. After diluting 1:10 with NET, the sera were added to obtain the appropriate dilution for immunoblots and reacted at 4°C overnight.

Immunofluorescence (IF) Studies
IF studies were carried out in 8 ep patients and in one patient with an ev-like syndrome associated with HPV3-induced plane and intermediate warts. Five patients contained papillomas with ev-specific HPVs and four with HPV3. One biopsy of an HPV2-induced common wart in a non-ev patient was also included as control. The data on the HPV typing in these patients by DNA hybridization and restriction enzyme cleavage were published previously [19,20].

Indirect IF (IIF) studies were performed with the use of immune serum against HPV8 L1-galactosidase fusion protein. A papillomavirus genus-specific rabbit immune serum against SDS disrupted HPV1 viromes and guinea pig immune sera against full virial particles isolated from HPV3- or HPV2-induced warts [21] were used as controls.

Biopsies of skin lesions were frozen in liquid nitrogen and embedded in OCT compound embedding medium. Consecutive sections (6–7 μm) were cut in a cryostat (Reichert-Jung) at −20°C and fixed for 10 min in cold acetone (−20°C). After rinsing in phosphate buffered saline (pH 7.2) for 20 min sections were incubated for 30 min at room temperature with appropriate dilutions of immune sera (Table I), rinsed twice for 10 min in phosphate buffered saline, and then incubated with diluted conjugates for another 30 min. After last washing (2 × 10 min) sections were mounted in buffered glycerol containing 1 mg/ml p-phenylenediamine (PPD) and examined under a Zeiss (Opton) fluorescence microscope.

RESULTS
Expression of a Cro-β-gal-L1 Fusion Protein
The procarotc expression vector pEX2 carries parts of the lambda cro gene and the E. coli lac Z gene, driven by the heat inducible P_6 promoter of lambda. A multiple cloning site exists at the 3’end of the lac Z gene. The 600 bp HaeIII fragment of HPV8, which encodes 200 amino acids from the central part of the major capsid protein, was inserted by blunt end ligation into the SmaI site of pEX2 (Fig 1). After transformation of E. coli POP2136 clones were identified, which contained the plasmid pEX2-L1. When incubated at 42°C these clones produced a protein with a molecular mass of 139,000, which was absent from cell lysates of control cultures grown at 28°C (Fig 2). The size of this protein corresponds to that expected for a cro-β-gal-L1 fusion protein.

Induction of a L1-specific Antiserum
The heat-inducible 139-kD protein was purified by preparative SDS-PAGE and used for immunization of two guinea pigs. The animals were bled after two booster injections when they showed titers of 1:10,000 and 1:50,000, respectively, in a β-galactosidase-specific ELISA (data not shown). The sera detected the fusion protein and β-galactosidase in a Western blot. After removing the anti-cro-galactosidase activity by absorption with a lysate of heat-induced pEX2-positive bacteria, the sera reacted only with the fusion protein (Fig 2). They revealed insert-specific titers of 1:7,500 and 1:25,000 in the Western blot.

Test for Serum Specificity
To test for the reactivity with authentic viral proteins, we used Western blots of protein extracts from an HPV1-induced planar wart and an HPV8-positive wart of an ev patient. The anti-L1-β-gal sera detected a 59-kD protein in the extract of the ev lesion but failed to react with proteins from the planar wart (Fig 3). Both preparations were known to contain virus capsid protein, which reacted with antibodies from patient’s sera (see below). The sizes of 56 and 59 kD corresponded to those calculated for L1 proteins of HPV1 and HPV8 from the respective nucleotide sequences [22,23].

For further characterization the guinea pig anti L1-β-gal sera were incubated with HPV8 viruses at a dilution of 1:10 and 1:50. Examination in the electron microscope showed separate particles with clearly visible capsomers. In contrast, agglutinated virions with a fuzzy surface were observed after control incubation with the serum of an ev patient at a dilution of 1:10. This result indicates that antisera against the engineered protein are not able to react with native virus particles.

<table>
<thead>
<tr>
<th>Table I. Characteristics of Immune Sera and Conjugates</th>
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<tbody>
<tr>
<td><strong>Immune sera against</strong></td>
</tr>
<tr>
<td>HPV8 L1-galactosidase fusion protein raised in guinea pig</td>
</tr>
<tr>
<td>SDS disrupted viral particles (group-specific) raised in rabbits</td>
</tr>
<tr>
<td>HPV3 full viral particles raised in guinea pig</td>
</tr>
<tr>
<td>HPV2 full viral particles raised in guinea pig</td>
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<table>
<thead>
<tr>
<th>Conjugate</th>
<th>mg-specific antibody/ml</th>
<th>F/P molar</th>
<th>Optimal dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goat anti-guinea pig IgG (Sigma)</td>
<td>1</td>
<td>3.4</td>
<td>1/40</td>
</tr>
<tr>
<td>Goat anti-rabbit IgG (Sigma)</td>
<td>1</td>
<td>3.6</td>
<td>1/40</td>
</tr>
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</table>
Immunofluorescence Studies of Biopsies from Benign Lesions of EV Patients

The anti-HPV8-L1-galactosidase antisera stained nuclei in the upper epidermal layers of red plaques of ev patients infected with HPV8 or other ev-specific HPV's (Fig 4 and Table II). All lesions positive for HPV3 and one HPV2-induced common wart from a non-ev patient proved to be negative with the use of L1 immune serum. The sensitivity of the described immune serum was higher than that of the genus-specific immune serum, i.e., the staining was stronger, with no background. In one patient negative for HPV antigen in one biopsy, the repeated biopsy was found to be positive with the L1-fusion protein antisera and weakly positive with the genus-specific antisera.

Test for Reactivity of Human Sera

Sera from four patients with ev-like lesions were analyzed for HPV-specific antibodies. Western blots of a plantar wart and an ev-specific papule served as antigen.

DISCUSSION

To obtain large quantities of HPV8-specific capsid antigen we cloned a 600-bp HaeIII fragment from the central part of ORF L1 into the procaryote expression vector pEX2. Transformed E. coli cells produced a 139-kD cro-β-galactosidase-HPV8 L1 fusion protein, which was used for immunization of guinea pigs and tested for reactivity with human sera.

The guinea pig antiserum against the engineered protein detected non-aggregated HPV8 capsid protein in Western blots and in indirect immunofluorescence tests with thin sections of ev biopsies. In Western blots the sera showed HPV8 specific titers of 1:25,000 and 1:7,500, respectively. The raised antisera were, however, not able to agglutinate native HPV8 virions, which suggests that relevant antigenic amino acid sequences are either masked or folded in a different way when capsomers are aggregated as virions. We assume that the expressed part of L1 is not exposed at the surface of virus particles because it does not seem to be immunoogenic in the course of the natural infection: Sera from ev patients, which were shown to detect the authentic HPV8 capsid protein in Western blots, failed to react with the L1 part of the fusion protein.

The guinea pig anti-HPV8-L1 antiserum also detected structural proteins of HPV5, HPV12, and HPV9, and HPV19, which are all associated with ev and related to HPV8 [2]. The recognition of these types in addition to HPV8 appears to be advantageous because ev patients are usually infected by several diverse ev-specific HPV's. The sera did not react with L1 proteins of HPV1, HPV2, and HPV3, thus showing subgroup specificity for ev-HPVs. Immunization with SDS denatured virus particles usually triggers a genus-specific immune response [24] that could have also been expected on the basis of our vaccination protocol. The subgroup specificity of our antisera may indicate that genus-specific antigenic determinants are not encoded within the 600-bp HaeIII fragment. A computer-assisted comparison of the L1 amino acid sequences of various papil-
Figure 4. Indirect immunofluorescence tests on the biopsies from patients with *Epidermodysplasia verruciformis* and controls. 

a. IIF with guinea pig anti-HPV8 L1 fusion protein at a dilution of 1:160 and fluorescein labeled goat anti-guinea pig IgG at a protein concentration of 1 mg/ml, molar F/P ratio 3.4. Biopsy from the patient with HPV8-induced ev. Viral capsid proteins are present in the upper layers of the epidermis. No background staining (×600). 

b. IIF with rabbit anti-common antigen at a dilution of 1:80 and fluorescein-labeled goat anti-rabbit IgG at a protein concentration of 1 mg/ml and molar F/P ratio 3.6. The same biopsy. Intense background staining (×600). 

c. IIF test with guinea pig anti-HPV8 L1 fusion protein as in a. Biopsy from patient JK with *Epidermodysplasia verruciformis* associated with numerous ev-specific HPVs. Viral capsid proteins present in the stratum corneum. No background staining (×600). 

d. IIF staining with guinea pig anti-HPV8 L1 fusion protein as in a and c. Biopsy from a common wart induced by HPV2. No detectable viral capsid proteins (×600). 

e. IIF test with guinea pig anti-HVP8 L1 fusion protein as in a, c, and d. Biopsy from patient TM with *Epidermodysplasia verruciformis* induced by HPV3. No detectable viral capsid proteins (×600). 

f. IIF test with guinea pig immune serum against capsidic antigen of HPV3 at a dilution of 1:160, and the same fluorescein-labeled goat anti-guinea pig IgG at a protein concentration of 1 mg/ml and molar F/P ratio 3.4. Biopsy from the same patient TM. Viral capsid proteins are present throughout the stratum granulosum (×600).
Table II. Immunofluorescence Study of Benign Lesions of ev Patients With the Use of Immune Serum Against L1 Major Capsid Protein of HPV8.

<table>
<thead>
<tr>
<th>Patients and diagnosis</th>
<th>Type of lesion</th>
<th>Type of HPV in biopsies&lt;sup&gt;a&lt;/sup&gt;</th>
<th>HPV8 L1 β-gal antigen</th>
<th>Common PV antigen</th>
<th>HPV3 capsid antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>DD&lt;sup&gt;a&lt;/sup&gt;, EV</td>
<td>Red plaque</td>
<td>HPV8</td>
<td>++/++</td>
<td>NT</td>
<td>W/+</td>
</tr>
<tr>
<td>RM, EV</td>
<td>Red plaque</td>
<td>HPV8</td>
<td>++/++</td>
<td>+</td>
<td>W/+</td>
</tr>
<tr>
<td>JK, EV</td>
<td>Red plaque</td>
<td>HPV12</td>
<td>−</td>
<td>−</td>
<td>NT</td>
</tr>
<tr>
<td>JD&lt;sup&gt;b&lt;/sup&gt;, EV</td>
<td>Red plaque</td>
<td>HPV5</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>KD, EV</td>
<td>Red plaque</td>
<td>HPV9</td>
<td>++</td>
<td>W/+</td>
<td>NT</td>
</tr>
<tr>
<td>TM, EV</td>
<td>v. plana like</td>
<td>HPV3</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>EB, EV</td>
<td>v. plana pigmented</td>
<td>HPV3</td>
<td>−</td>
<td>−</td>
<td>++</td>
</tr>
<tr>
<td>RK&lt;sup&gt;c&lt;/sup&gt;</td>
<td>v. plana</td>
<td>HPV3</td>
<td>−</td>
<td>⊕</td>
<td>++</td>
</tr>
<tr>
<td>widespread warts</td>
<td>v. intermediate</td>
<td>HPV3</td>
<td>−</td>
<td>⊕</td>
<td>++</td>
</tr>
</tbody>
</table>

<sup>a</sup> Data on HPV typing were published previously [19, 20].

<sup>b</sup> These patients had mixed infection with ev-specific HPVs and HPV3. The study was performed in red plaques induced by ev-specific HPVs.

<sup>c</sup> In this 52-year-old patient, infected since childhood with HPV3, red plaques started to appear on covered sites of the body.

<sup>d</sup> In this patient, widely disseminated plane and intermediate warts appeared at the age of 7, and therefore she is classified as ev-like syndrome associated with HPV3.

<sup>e</sup> W: weak positive

<sup>f</sup> NT: Not tested.

Lomavirus confirms that both amino- and carboxysterminus are highly conserved, whereas the central part reveals greater variability (Fig 6). A stretch of 40 amino acids exists within the expressed segment, which differs completely among the known L1 sequences. The experience of this work suggests that corresponding sequences of ORF L1 should be expressed for the production of type-specific antibodies against other HPVs. The alternative could be sequences of the L2 protein, which is highly type-specific in its carboxyterminal part. This capsid component is, however, expressed in much smaller amounts.

Subgroup specificity for ev associated viruses was not only observed with the guinea pig antisera but also with sera from ev patients. Both the serum of an HPV5- and HPV46-infected patient reacted with HPV8 L1. The major capsid proteins of HPV5 and HPV8 show 90% amino acid sequence homology (Fig 6) and considerable relationship even in the central part of the molecules.

The antisera described in this paper present important diagnostic reagents for immunofluorescence detection of ev-specific HPVs in biopsies of benign lesions. Whereas typical ev is easily diagnosed, there are abortive cases with a few red plaques on the covered sites of the body which are, not infrequently, overlooked. The described test may be most helpful for recognition of such cases and for sporadic lesions in transplant recipients and other immunosuppressed patients. The clinical and histologic features of cutaneous lesions in these patients are often non-characteristic, and there is an increasing body of evidence of their association with ev-specific HPVs [4–8]. Thus this simple immunofluorescence test with L1 structural protein antisera should find wide application in clinical practice.

Table III. HPV Specific Antibodies in Sera From ev Patients

<table>
<thead>
<tr>
<th>Patient and diagnosis</th>
<th>Antibodies against the major capsid protein of</th>
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<tbody>
<tr>
<td>E.D. ev/Hodgkin’s disease</td>
<td>HPV46</td>
</tr>
<tr>
<td>P.S. ev/Hodgkin’s disease</td>
<td>HPV46 related</td>
</tr>
<tr>
<td>G.D. ev</td>
<td>HPV5</td>
</tr>
<tr>
<td>C. ev</td>
<td>HPV5, 8, 19, 20, 25</td>
</tr>
</tbody>
</table>

<sup>a</sup> Typing was performed by DNA hybridization and restriction enzyme cleavage analysis.

Figure 5. Western blots of protein extracts from an HPV1 induced wart (lane 2), an HPV8-induced lesion (lane 1), and E. coli POP2136 cells, transformed with pEX2 (lane 3), pEX2/L1 (lane 4). Proteins were separated by 8% (D) or 12.5% (A–C) SDS-PAGE and transferred onto nitrocellulose filters. The filters were incubated with sera of patients (A) E.D., (B) P.S., (C) G.D., and (D) C. Filters containing the L1-β-gal fusion protein were also treated with serum of patient G.D. after absorption with an E. coli (POP2136 pEX2) lysate (CI). Arrowheads indicate the position of the protein band detected by the sera.
REFERENCES


