Identification of Immunogenic Regions of the Major Coat Protein of Human Papillomavirus Type 16 that Contain Type-restricted Epitopes

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SUMMARY

We have identified regions of the major capsid protein, L1, of the human papillomavirus (HPV) type 16 (HPV-16 L1), that are recognized by five monoclonal antibodies (MAbs) raised to a bacterial fusion protein containing residues 172 to 375 of HPV-16 L1. All five MAbs recognized HPV-16-infected tissue sections by immuno-histochemistry, but not sections infected with HPV-1a (cutaneous warts), HPV-6b or -11 (genital warts). MAbs 3D1, 5A4 and 1D6 also recognized HPV-2-infected sections (cutaneous warts); MAb 8C4 recognized only sections containing HPV-16. Four MAbs (8C4, 3D1, 1D6 and 5A4) recognized a synthetic peptide corresponding to residues 269 to 284 of HPV-16 L1; within this region a minimum antibody binding site was identified, a tripeptide 276 to 278. However the complete epitope appears to extend beyond these residues and beyond HPV-16 L1 (269 to 284). The fifth MAb, 1C6, recognized bacterial fusion proteins containing HPV-6b L1, -16 L1 or -18 L1 using immunoblots, yet appeared HPV-16-specific when tested on infected tissue sections. This MAb recognized five amino acids within a different region of HPV-16 L1 (residues 299 to 313).

INTRODUCTION

Papillomaviruses infect avian, reptile and mammalian hosts, causing epithelial proliferation (Sundberg, 1987). Humans are susceptible to at least 58 types of papillomaviruses. Human papillomavirus (HPV) types 16, 18, 31, 33 and 52 which cause genital infections have been associated with high grade cervical intraepithelial neoplasia lesions and invasive carcinoma, and other genital HPV types (e.g. HPV-6b and HPV-11) are more commonly associated with benign conditions such as genital warts (McCance, 1986). If the relationship between certain types of HPV and pre-invasive and invasive cancer of the cervix is proven to be of a causal rather than casual nature, rapid and accurate diagnosis of the type of HPV infection is likely to be important in the clinical management of patients with these diseases. At present, diagnosis of the exact type of HPV infection relies upon nucleic acid hybridization techniques, which are time-consuming and expensive when compared to immunohistochemical or serological methods of viral identification. A serological test for exposure to high risk HPV types would thus provide an easier method of identifying patients who may be at greatest risk of developing a malignant disease.

The major capsid protein (L1) of papillomaviruses, which is the most abundant virus-encoded protein, is highly conserved between different types, with amino acid homologies as high as 80% existing between some L1 proteins from different papillomavirus types. As a consequence...
serological typing of papillomaviruses has been unsuccessful. In addition, the availability of native HPV proteins is limited as papillomaviruses cannot be propagated in vitro (Taichman et al., 1984) and genital lesions contain few assembled virions. However, using molecular biological techniques it is possible to produce substantial quantities of recombinant proteins containing HPV amino acid sequences (Doorbar & Gallimore, 1987; Tomita et al., 1987; Banks et al., 1987; Browne et al., 1988; Patel et al., 1989).

Bacterial fusion proteins, consisting of β-galactosidase and part of the L1 protein of HPV-16, have been used to produce MAbs for histological diagnoses of HPV-16 in cervical biopsies (Patel et al., 1989). The immune response of a mouse to the HPV-16 L1 fusion protein may differ from that of humans infected with intact HPV-16 virions, but epitopes recognized by monoclonal antibodies (MAbs) may also be recognized by humans. Such subregions of HPV-16 L1 may prove to be useful targets for identifying patients exposed to this virus and thus for seroepidemiological studies. The current report describes the identification and characterization of MAb epitopes using synthetic peptides, with a view to assessing the usefulness of peptides as targets to detect HPV-16 antibodies amongst patients with cervical intraepithelial neoplasia.

**METHODS**

Recombinant HPV proteins. β-Galactosidase fusion proteins of HPV-16 L1 [BamHI (nucleotide 6150) and PstI (6787)] and HPV-6b L1 [XbaI (5903) to PstI (6489)] were produced using pEx plasmid vectors (Stanley & Luzio, 1984). Dr D. Galloway (Fred Hutchinson Cancer Research Center, Seattle, Wash., U.S.A.) provided other L1 open reading frame DNAs cloned into PATH II expression vectors: HPV-6b L1 [XbaI (5903) to HpaII (7862)]; HPV-16 L1 [BsrNI (5529) to NsiI (253)] and HPV-18 L1 [XbaI (5730) to XbaI (321)]. These were grown in Escherichia coli to produce tryptophan E synthetase-HPV L1 fusion proteins.

**Peptides.** Twelve peptides corresponding to overlapping subregions of HPV-16 L1 (residues 172 to 375) and HPV-16 L1/HPV-16 L1 heteropeptides (see below) were synthesized (Houghten et al., 1980; Sutcliffe 1980; Margalit & Merryfield, 1970) and purified by HPLC, and five other peptides were obtained from Dr J. Rothbard (Imperial Cancer Research Fund, London, U.K.). The correct biochemical composition of peptides of particular interest (269 to 284 and 299 to 313) were checked by Drs C. R. Howard and A. Frew (London School of Hygiene and Tropical Medicine, London, U.K.). In order to examine the fine detail of epitopes other peptides were synthesized onto polyethylene pins using an epitope scanning kit (Cambridge Research Biochemicals).

Fig. 1 illustrates the relationship of the synthetic peptides and HPV fusion proteins used in the present study.

**MAbs.** Murine hybridomas were produced (Köhler & Milstein, 1975) by fusing NS1/1.Ag4.1 myeloma cells and spleen cells from a female BALB/c mouse immunized with HPV-16 L1 (172 to 375) as described by Patel et al. (1989). Five MAbs (8C4, 3D1, 1D6, 5A4 and 1C6) were included in the present study. MAb isotypes were determined by immunodiffusion (Serotec).

Reactivity of MAbs with L1 proteins and peptides. Three techniques were used to characterize MAb specificities.

(i) MAb recognition of tissue sections known to contain DNA of HPV-6b, HPV-11, HPV-16 or HPV-18 by the immunoperoxidase technique on formaldehyde-preserved sections and, for HPV-1a and HPV-2, by immunofluorescence on frozen tissue sections (HPV-1a and HPV-2 tests were performed by Dr J. Doorbar, University of Birmingham, U.K.). All tissue sections used were from productive lesions in that they expressed papillomavirus type-common antigens (Jenssen et al., 1980) identified with rabbit antisera to disrupted bovine papillomavirus type 1 (BPV-1 particles (Dako) and to an HPV-16 L1 fusion protein (Patel et al., 1989). Both of these antisera cross-react with all HPV L1 proteins tested (Patel et al., 1989).

(ii) MAb reactivity against the HPV-6b, HPV-16 and HPV-18 fusion proteins was tested by immunoblotting as described previously (Patel et al., 1989; Towbin et al., 1979).

(iii) ELISA tests were carried out in 96-well plates (Nunc) coated with 90 μl/well of HPV-16 L1 (amino acids 172 to 375) (0.26 mg/l) in phosphate-buffered saline (PBS) for 1 h at room temperature, washed three times with PBS–TWEEN-20 (0.2%, v/v; BDH) (or for screening assays tap water; Ashorn & Krohn, 1986) and then incubated with 90 μl/well of bovine serum albumin (BSA; 1 g/l PBS) for 1 h. Plates were washed and exposed to 50 μl/well of MAb, or 40 μl MAb peptide mixture (see below), and then incubated overnight at 4 °C. Plates were washed again and 50 μl/well of horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin (Dako; 1:500 in PBS) was added for 1 h at room temperature. Plates were washed and the substrate was added [50 μl/well of a solution containing 61.7 mm-diam monosodium orthophosphate, 26.6 mm-citric acid, 11 mm-o-phenylene diamine and 0.5 ml/l hydrogen peroxide (100 volumes)]. Reactions were terminated with 25 μl/well of 2 m-sulphuric acid and absorbances (490 nm) were determined on a Dynatech MR-700 plate reader.

Screening assay to detect antigenic regions of HPV-16 L1 to fusion protein with MAb. Antigenic regions of HPV-16 L1 were detected by assessing the inhibitory effect of 20 μl of peptide (at 10 mg/l PBS) upon the binding of 20 μl of...
Fig. 1. Relationship of fusion proteins and synthetic peptides to the HPV-16 L1 product. Numbering (from the first methionine residue) in all cases relates to the relative amino acid position on HPV-16 L1 to which the aligned peptide or protein corresponds. Left and right represents NH$_2$ and COOH termini.
MAb (1:20 in PBS) to HPV-16 L1 (172 to 375)-coated plates in ELISA; all samples were assayed in triplicate on at least two occasions. When inhibition was detected, a detailed investigation was performed by measuring the effects of different concentrations of peptide upon titration curves of the MAb. In such instances, 40 µl/well of an equal mixture of peptide (10, 1, 0.1 or 0.01 mg/l PBS) and MAb (at dilutions of 1:2 to 1:4096 in PBS) were assessed by ELISA. In each experiment a reference peptide, not recognized by any of the MAbs in screening assays (HPV-16 L1, 329 to 343) was included.

When a particular peptide was recognized by a MAb, the binding site was examined further by synthesizing peptides with an epitope scanning kit. MAb reactivity (at 1:20 dilution, overnight at 4 °C) towards these peptides was determined using published methods involving a direct ELISA system (Geyseren et al., 1987). For each set of synthesized peptides produced, a positive (PLAQ) and negative (GLAQ) quadrameric peptide control was synthesized and assayed against a test MAb (supplied by Cambridge Research Biochemicals).

Antibody affinities. MAb dissociation constants (Kd), were determined by measuring free MAb (by direct ELISA) present after a 15 h incubation at 4 °C of mixtures of MAb (1:20) with an equal volume of peptide (44.8 × 10⁻⁶ to 0.0088 × 10⁻⁶ M in PBS containing 10 g/l BSA. Kd values were calculated from data points within the linear portion of inhibition curves (Friguet et al., 1985).

Analyses of HPV-16 L1 from sequence data. A hydrophilicity chart (Hopp & Wodds, 1981) and Kabat and Wu amino acid variability plot (Roitt et al., 1985) at each residue were constructed from aligned putative amino acid sequences of HPV proteins (Baker, 1987). Other amino acid sequences were obtained from J. Doorbar (HPV-2 L1) and published sequences of HPV-11 (Dartmann et al., 1986) and HPV-18 (Cole & Danos, 1987). Alignment of amino acid sequences of HPV-1a L1, HPV-6b L1 and HPV-16 L1 was taken from Baker (1987) and of HPV-2 L1, HPV-11 L1 and HPV-18 L1 with HPV-16 L1, by the position of common motifs at the region of interest. Amino acid nomenclature in the present report follows the single letter code, agreed by the IUPAC-IUB Joint Commission on Biochemical Nomenclature in 1968.

Statistical tests. Unpaired Student's t-tests were used to assist the interpretation of MAb affinity data.

RESULTS

MAb isotypes and specificities

All MAbs were of the IgG1 isotype. MAb recognition of viral antigens in tissue sections known to contain DNA of different HPV types and to express conserved capsid antigens, revealed that all MAbs recognized HPV-16, whereas none recognized sections containing HPV-1a, -6b, -11 or -18. MAbs 3D1, 5A4 and 1D6 also recognized HPV-2-infected sections, but MAb 8C4 recognized only HPV-16-infected lesions (Table 1 and Patel et al., 1989). MAb recognition of bacterial fusion proteins using immunoblots indicated that MAbs 8C4, 3D1, 5A4 and 1D6 were able to distinguish between the immunogen, HPV-16 L1 (amino acids 172 to 375), and also the full-length HPV-16 L1 (residues 1 to 505), from fusion proteins containing HPV-6b L1 (38 to 505), HPV-6b L1 (38 to 232) or HPV-18 L1 (100 to 505). MAb 1C6 recognized HPV-16 L1 (172 to 375) and 0 to 505), HPV-6b L1 (38 to 505) and HPV-18 L1 (1 to 505), but not HPV-6b L1 (38 to 232). None of the MAbs reacted with E. coli β-galactosidase (Sigma) or extracts from uninduced E. coli.

Identification of antigenic regions

To reveal MAb binding sites on the L1 protein, a series of overlapping peptides corresponding to HPV-16 L1 (172 to 375) were synthesized (Fig. 1). Screening assays, using a fixed dose of peptide to inhibit ELISA reactivity of a single dilution of MAb, indicated that MAbs 8C4, 3D1, 5A4 and 1D6 recognized peptide 269 to 284. In contrast, 1C6 recognized peptide 299 to 313 (Fig. 2). Confirmation of these data was provided by the concentration-dependent inhibition of the titration curves of MAbs 8C4, 3D1, 5A4 and 1D6 to HPV-16 L1 (172 to 375)-coated ELISA plates by peptide 269 to 284 (but not by peptide 329 to 343) (Fig. 3). Similar dose-dependent inhibition of MAb 1C6 by 299 to 313 (but not by 329 to 343) was also detected (data not shown). MAb 1C6 cross-reacted with HPV-6b and -18 fusion proteins in immunoblot tests, but not with HPV-6b-infected tissues, suggesting that this MAb had a more extensive spectrum of reactivity than the others and was therefore included as a control. Other MAbs against HPV-16 L1 (172 to 375) not included in the present report had characteristics similar to those of 1C6.
HPV-16 type-restricted epitopes

Fig. 2. Inhibition of MAb binding to HPV-16 L1 in ELISA tests by synthetic peptides. Each set of bars represents the mean (± 1 s.d.) of triplicate estimations of the binding of each MAb to HPV-16 L1 (172 to 375) in the presence of one of the overlapping peptides. Results in each instance are arranged in order from peptides 183 to 198 (left) to 361 to 375 (right) (order as in Fig. 1). Each set of data represents an individual experiment.

Table 1. Characteristics of MAbs investigated

<table>
<thead>
<tr>
<th>MAb</th>
<th>Isotype</th>
<th>Immunoblot reactivity</th>
<th>Biopsy*</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>6b</td>
<td>16</td>
</tr>
<tr>
<td>8C4</td>
<td>IgG1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3D1</td>
<td>IgG1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5A4</td>
<td>IgG1</td>
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<td>+</td>
</tr>
<tr>
<td>1C6</td>
<td>IgG1</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

* Biopsy: tissue sections of cutaneous warts, genital warts or cervical intraepithelial neoplasias infected with various HPV types were tested either by immunofluorescence (HPV-1a and -2) or by the immunoperoxidase (HPV-6b, -11, -16 and -18) technique. + Indicates recognition of the biopsy whereas - is negative.
† (a) Fusion protein containing HPV-6b L1 (amino acids 38 to 232); (b), HPV-6b L1 (38 to 505); (c), HPV-16 L1 (0 to 505); (d), HPV-16 L1 (172 to 375); HPV-18; HPV-18 L1 (100 to 505).
‡ ND, Not determined.

MAb affinities

As four MAbs recognized peptide 269 to 284 and all were IgG1, MAb $K_d$ constants were quantified to determine whether these MAbs were the products of four distinct cell lines. The MAb dissociation constants were dissimilar: 8C4 had a substantially lower $K_d$ value than the others; 8C4 and 5A4 were significantly different to one another and to 3D1 and 1D6; 3D1 and 1D6 could not be differentiated (Table 2). MAbs 3D1 and 1D6 were subsequently found to recognize different peptides and this is described later.
Fine mapping of epitopes

The specificity of MAbs 8C4, 3D1, 5A4 and 1D6 to HPV-16 L1 (e.g. as compared to HPV-6b L1) must depend upon residues unique to HPV-16 L1 (269 to 284). Inspection of the amino acid sequences of HPV-16 L1 and HPV-6b L1 at region 269 to 284 (Table 3) revealed that only the residues N...D.Y.....STA were unique to HPV-16 L1. Of these, it appeared likely that the sequence STA was important as these residues occur at positions which are hypervariable when aligned L1 proteins of different papillomaviruses are compared (Fig. 4). Thus, an HPV-6b L1 peptide corresponding to HPV-16 L1 (269 to 284) and four HPV-16–HPV-6b L1 heteropeptides were synthesized. These consisted of a series of HPV-16 L1 (269 to 284) peptides each containing a single insertion of a corresponding HPV-6b residue.
Table 3. Comparison of putative amino acid sequences over the regions of interest, 299 to 313 and 269 to 284 of HPV-16 L1

<table>
<thead>
<tr>
<th>HPV type</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>16 (299 to 313)</td>
<td>MVTSDAQIFNKPYWL</td>
</tr>
<tr>
<td>2</td>
<td>...MVSSSEQQLFNKPYWL...</td>
</tr>
<tr>
<td>6b</td>
<td>...LVSEAOFLFNKPYWL...</td>
</tr>
<tr>
<td>18</td>
<td>...IVTSDSQLFNKPYWL...</td>
</tr>
<tr>
<td>11</td>
<td>...LVSEAOFLFNFPYWL...</td>
</tr>
<tr>
<td>16 (269 to 284)</td>
<td>ENVPDDLYIKGSGSTA</td>
</tr>
<tr>
<td>2</td>
<td>...DTIPDELYIKSFPVT...</td>
</tr>
<tr>
<td>6b</td>
<td>...EPVPDTLIIKGSNRT...</td>
</tr>
<tr>
<td>18</td>
<td>...DTPQSLSLYIKTGMR...</td>
</tr>
<tr>
<td>11</td>
<td>...EVVDLLVKGANNR...</td>
</tr>
<tr>
<td>1a</td>
<td>...EAVPQSLYLTADAEPR...</td>
</tr>
</tbody>
</table>

Inhibition ELISAs indicated that only the HPV-16 L1 heteropeptide containing an isoleucine (I) substitution for a tyrosine (Y) residue at position 276 (i.e. peptide ENVPDDLIIKGSSTA) and the complete HPV-6b L1 peptide were unable to inhibit (i.e. the peptides were not recognized by) MAbs 8C4, 3D1, 1D6 and 5A4 recognition of plates coated with HPV-16 L1 (172 to 375) (Fig. 5). Although for MAb 5A4 the HPV-6b L1 peptide was slightly (and consistently) more inhibitory than the HPV-16 L1 heteropeptide containing the isoleucine for tyrosine substitution at position 276, this difference was not observed when other MAbs (8C4, 1D6 and 3D1) were tested (Fig. 5). These data indicate that HPV-16 specificity of the MAbs does not depend upon the terminal STA sequence, but that the tyrosine (Y) residue at position 276 (of HPV-16 L1) is critical (Table 3).

Using the epitope scanning kit, 11 overlapping hexapeptides of HPV-16 L1 between residues 269 and 284 (ENVPDDLIIKGSSTA) were synthesized. MAbs 1D6 and 3D1 recognized the sequence DDLYIK (273 to 278; predominant), but also DLYIKG, LYIKGS and YIKGSG. MAbs 5A4 and 8C4 reacted with all but one (DLYIKG) of these peptides. Concordant results were obtained from three separate syntheses of these peptide sets (representative data: Fig. 6) and the MAbs did not recognize any of 15 control hexapeptides containing random amino acid sequences (data not shown). In order to identify the minimum antigenic sequence in contact with antibody three scans of overlapping penta-, quadra- and tripeptides of region 273 to 281 (DDLYIKGSG) were produced; significant binding of 8C4, 3D1, 1D6 and 5A4 was conserved when the peptide target was sequentially reduced to the tripeptide YIK (Fig. 6).

Interestingly, assay of hexapeptides of cottontail rabbit papillomavirus (CRPV), BPV-1, HPV-1a, HPV-2, HPV-6b, HPV-11 and HPV-18 corresponding to region 273 to 278 of HPV-16 L1 (i.e. that hexapeptide predominantly recognized by all four MAbs) revealed that although all MAbs were non-reactive with HPV-6b (DTLIIK) and HPV-11 (DDLLYK), they all recognized HPV-2 (DELYIK) and HPV-18 (QSLYIK) in addition to HPV-16 (DDLYIK) (data not shown). Furthermore, MAb 1D6 recognized the BPV-1 peptide (TDFYIK), 3D1 recognized CRPV (SRAYIK) and BPV-1, but 8C4 recognized neither of these (data not shown). Hence MAbs 1D6 and 3D1 can be distinguished by their peptide binding characteristics but not by $K_d$ value. Whilst the hexapeptide studies indicated MAbs reacted with several sequences not belonging to HPV-16 MAbs were HPV-16-specific (8C4) or HPV-16/HPV-2 restricted (1D6, 5A4 and 3D1) when tested by immunochemistry on infected tissue sections (Table 1).

MAb binding sites on HPV-16 L1 can be mapped therefore to the YIK trimer, and this explains the lack of reactivity of the MAbs with HPV-6b and -11, but the complete epitopes must extend beyond this to incorporate regions of HPV-16 L1 that are dissimilar to HPV-2 and HPV-18, since the MAbs can distinguish between HPV-18 L1 fusion proteins and HPV-18-infected tissues from HPV-16 L1 fusion proteins and infected tissues.

To identify such outlying, but structurally important, residues, seven overlapping decapetides incorporating the YIK motif were synthesized for HPV-16 L1 (between residues 269 and 284) and corresponding decamer scans of HPV types 1a, 2, 6b, 11 and 18. Analysis of
Fig. 4. Features of HPV-16 L1 (172 to 375) derived from the primary structure. (a) Variability of amino acids (Kabut & Wu plots); sequences for CRPV, BPV-1, HPV-1a and HPV-6b were compared to the HPV-16 sequence. Beneath the variability plot (unlabelled) are the positions of amino acid homology for aligned sequences of HPV-16 L1 and HPV-6b L1. Solid bars, 269 to 284 and 299 to 313 indicate the two regions recognized by MAbs in the present study. (b) Hydrophilicity plot (Hopp & Woods algorithm) of HPV-16 L1; data are expressed as the average hydrophilicity at the first residue of each hexameric window. Hydrophilicity plots for regions on L1 of HPV-1a, -2, -6b, -11 and -18 concordant with region 267 to 313 of HPV-16 L1 are included for comparison.
these peptides indicated that all four MAbs recognized all peptides containing HPV-2, -16 and -18 sequences (i.e. containing YIK), but not those corresponding to HPV-1a, -6b or -11 (without YIK) (data not shown). Indeed, even 16-mer peptides of HPV-16 L1 (269 to 284) and corresponding regions (see Table 3) of HPV types 2 and 18, but not of 6b or 11, were recognized by the MAbs (Fig. 7).

A similar, though less detailed, hexapeptide scan analysis of the epitope of MAb 1C6 indicated a major binding region within amino acids 299 to 313 to be a highly conserved segment, 308 to 312 (FNKPYW), of HPV-16 L1 (data not shown). However, MAb 1C6 did not recognize tissue sections containing HPV-2 or the HPV-6b L1 peptide 301 to 313 (SSEAQLFNKPYWL) (data not shown) which both contain the FNKPYW motif (Table 3).

**DISCUSSION**

The present study describes the characterization and epitope mapping of a series of MAbs raised against a fusion protein containing part of the L1 protein of HPV-16. There are 58 types of HPV and the genital HPV types 6, 11, 16 and 18 have a very high degree of amino acid homology (e.g. in aligned sequences of HPV-16 L1 with HPV-6b L1, 68% of the residues are identical; comparisons between L1 of HPV-6b and HPV-11, and between L1 of HPV-16 and HPV-18, reveal homologies approaching 80%). Despite this high degree of conservation, we have produced MAbs that recognize regions of a β-galactosidase–HPV-16 L1 (172 to 375) fusion protein and which have a limited cross-reactivity with L1 proteins of other HPV types.

One MAb (8C4) was HPV-16-specific when tested on tissue sections of HPV-2, -6b, -11 and -18; three others (3D1, 5A4 and 1D6) cross-reacted only with tissues containing HPV-2. Such HPV type-restricted MAbs may be of value in distinguishing those genital HPV types which are associated with cervical carcinoma (HPV-16, -18) and those which are not (HPV-6b, -11). HPV-2 rarely infects genital tissues and is not usually associated with the development of malignant lesions, hence cross-reactivity with HPV-2 may be irrelevant with respect to immunohistochemical identification with MAbs of high-risk HPV types in genital mucosa.
Fig. 6. Scanning for epitopes and minimal antibody binding regions within the HPV-16 L1 region 269 to 284 utilizing the epitope scanning kit in direct ELISAs. Left (NH₂ terminus) to right (COOH terminus). A: hexamers within HPV-16 L1 (269 to 284); pentameric (B), quadrameric (C) and trimeric (D) scans of region 273 to 281. The data set for each MAb shows representative results of a single assay performed on the same occasion.
Papillomavirus antigens in the nuclei of cells from patients with premalignant disease of the cervix are currently detected using antisera to disrupted BPV-1 (Kurman et al., 1981) or to pooled wart preparations (Walker et al., 1983). Several groups have produced antisera to specific HPV L1 proteins. Polyclonal antisera have been raised against HPV-1 L1 (Doorbar & Gallimore, 1987), to HPV-6b L1 (Tomita et al., 1987) and to HPV-16 L1 fusion proteins and synthetic peptides (Banks et al., 1987; Browne et al., 1988; Patel et al., 1989). Some of these sera
are apparently type-specific; for example, antisera against an HPV-16 L1 fusion protein (Browne et al., 1988) does not recognize an HPV-6b L1 fusion protein, but antibodies against HPV-6b L1 (Tomita et al., 1987) cross-react with the major capsid protein of HPV-1 (Li et al., 1987). These cross-reactivities have been mapped to the mid-portion of the L1 protein, as fusion proteins expressing HPV-6b L1 (amino acids 75 to 234 and 234 to 432) are recognized by antisera to BPV-1 and vice versa (Strike et al., 1989). However, many of these sera have not been tested extensively against L1 proteins from different HPV types nor have they been tested for reactivity against HPV-infected tissue sections. Thus there is a requirement for a panel of well-characterized MAbs specific to HPV-16 for the immunohistochemical identification of HPV-16 antigens in tissue samples. Although the MAbs described in the present study were not truly HPV-16 type-specific in all tests, they were capable of identifying a restricted number of L1 types in infected tissues.

For reasons discussed earlier, it was not possible to test the MAbs directly against intact HPV-16 virions, and thus it is not known whether the MAb epitopes described above are exposed on intact virions. MAb reactivity toward tissue sections may represent the recognition of intact virus particles; however, it may also indicate MAb binding to either proteins in the process of assembly into virions, or to linear L1 proteins. Furthermore, as no X-ray crystallographic data on L1 of HPV-16 (or indeed any papillomavirus capsid protein) have been published it is not known whether the MAb epitopes identified in the present study are internal or external.

The HPV-16 L1 epitopes recognized by MAbs 8C4, 1D6, 3D1 and 5A4 were initially mapped to amino acids 269 to 284 and that of MAb 1C6 to amino acids 299 to 313. Using HPV-16/HPV-6b heteropeptides and an epitope mapping kit, the antigenic determinant recognized by MAbs 8C4, 1D6, 3D1 and 5A4 was sequentially reduced from region 269 to 284 to a tripeptide, YIK (HPV-16 L1, 276 to 278). Even the substitution of a single residue, isoleucine (I) for the tyrosine (Y) residue at position 276 of the HPV-16 L1 peptide 269 to 284, was sufficient to prevent MAb recognition. This factor alone probably underlies the lack of recognition of HPV-6b and HPV-11 by these MAbs as they contain isoleucine (HPV-6b) or leucine (HPV-11) at comparable positions. Indeed, use of a computer molecular modeling program (Crabbe & Appleyard, 1989) to estimate the structure of the peptides revealed that substitution of isoleucine for tyrosine at position 276 of peptide 269 to 284 of HPV-16 L1 significantly altered the symmetrical turn in the native HPV-16 sequence (Fig. 8a and b). In contrast, HPV-16 L1 (299 to 313) was predicted to be a linear peptide (Fig. 8c).

Three MAbs (1D6, 3D1 and 5A4) reacted with tissues infected with HPV-16 and also those infected with HPV-2, whereas MAb 8C4 identified only HPV-16. These data suggest that at least two epitopes have been identified. Interestingly, the four MAbs recognized peptide 269 to 284 and also the same minimal antibody binding site, the trimer YIK, suggesting that they may be identical sibling B cell clones generated in vivo from a common maternal precursor. Indeed, all MAbs included in the present study were produced from a single fusion experiment and all were of the IgG1 isotype. However all MAbs that recognized peptide 269 to 284 were distinguishable either on the basis of their respective $K_d$ values or on their differing patterns of binding to peptides of BPV-1 L1, CRPV L1 or HPV-16 L1 (274 to 279).

The sequence YIK is probably in intimate contact with the MAb antigen-binding sites, but this motif is common to several types of papillomavirus (with the notable exceptions of HPV-6b and HPV-11). It occurs in HPV-2 L1 and HPV-18 L1, which are not recognized by MAb 8C4 when presented as a virus in situ or as an intact fusion protein, respectively. Paradoxically, however, MAb 8C4 recognizes hexameric HPV-2 and HPV-18 peptides corresponding to HPV-16 L1 (273 to 278). Thus the complete epitopes of these MAbs may extend beyond this region to include other critical residues. Although larger (decamer and 16-mer) peptides were assembled, these constructs failed to explain the differences in MAb reactivities against HPV-2 and HPV-18 hexapeptides, and HPV-2 tissue sections or HPV-18 fusion proteins, noted earlier.

Ambiguities therefore exist between the reactivity of the MAbs against viral proteins in tissues, to fusion proteins and to synthetic peptides. For instance, MAb 1D6 reacted with peptides corresponding to HPV-18 amino acid sequences, but not to an HPV-18 L1 bacterial fusion protein. The differential response of MAbs against viral proteins in situ and bacterial
fusion proteins may be explained by the lack of post-transcriptional modification in proteins expressed in bacterial systems (Treston & Mulshine, 1989). Alternatively, the differing methods of fixation (formaldehyde and frozen sections) and identification (immunoperoxidase and immunofluorescence) of viral antigens in tissue sections, conducted in different laboratories, may have differentially affected the preservation of similar antigens in different HPV types. For example, the Y1K motif may have been preserved in the frozen HPV-2 tissue sections, but not in formaldehyde-fixed tissues containing HPV-18. This explanation is unlikely, because the Y1K motif in formaldehyde-fixed HPV-16-infected tissues is recognized. There were also ambiguities in the reactivity of some MAbs to peptides. Indeed, MAb 5A4 and 8C4 recognized the sequence DDL1YIK and LYIKGs, but not DLYIKG. The reason for this is unknown, but it probably relates to stereochemical differences; however these were not apparent from examining computer-predicted structures (data not shown). One possible explanation for these discordant data is that the conformation of small peptides, bound to an inert matrix (as in the epitope mapping kit), will to some extent be limited by the attachment. Epitopes contained in such constructs may thus be internalized and unavailable for recognition by MAbs.

Therefore we suggest that although discrete regions of an epitope can be mapped readily using synthetic peptides, the complete antigenic structure of apparently linear epitopes frequently includes distant residues. These may be either distant residues which are in contact with the variable region binding site of a MAb (i.e. they form part of a discontinuous epitope) and/or may be adjacent regions which contribute to the overall secondary/tertiary structure of the site recognized by an antibody. Such flanking regions may be of paramount importance in determining whether a sequence, which when in the form of a small peptide can be recognized by an antibody, is exposed in an antigenic form on intact proteins or the virus in situ. Such a phenomenon would be missed in studies that rely solely upon peptide mapping data to define MA b epitopes.

The high frequency of HPV-16 type-restricted MAbs directed against region 269 to 284 of L1 is difficult to explain. However, this region contains the most hydrophilic peak of HPV-16 L1 (172 to 375), and thus may be immunodominant (Hopp, 1986). In addition, on comparing the sequences of other L1 proteins, 269 to 284 contains a cluster of hypervariable residues (Fig. 4). In contrast, the region recognized by the more broadly reactive MAb 1C6 (299 to 313) of HPV-16 L1 is highly conserved and less hydrophilic (Fig. 4). Furthermore, hydrophilicity analysis of HPV-16 L1 using a predictive algorithm (Hopp & Woods, 1981) indicates a high degree of similarity between HPV-16 and HPV-2 L1 proteins over the region of interest (269 to 284) in comparison to other HPV L1 proteins used in the present study (Fig. 4) and may account for the cross-reactivity of several of the HPV-16 MAbs with HPV-2-infected tissues.

Other hypervariable and hydrophilic regions on HPV-16 L1 (172 to 375) exist, most notably around residue 349; yet they did not induce HPV-16-specific MAbs. However gross differences exist between the response of murine strains (Morrow et al., 1983) and individuals within responder strains in their ability to produce antibodies to distinct peptide subregions of a protein immunogen. This latter phenomenon may be explained by a random selection of B cell clones from a much larger potential repertoire (Benjamini et al., 1988).

In conclusion, we have defined the binding sites of MAbs on HPV-16 L1 but, for reasons discussed above, not their complete epitopes. Nevertheless, regions 269 to 284 and 299 to 313 of papillomavirus L1 proteins are worthy of detailed investigation as potential targets for HPV type-restricted antibody tests. We are currently evaluating a variety of peptides as immobilized targets to detect antibodies to HPV-16 in a serological test. Such an essay would permit the rapid and accurate screening of large patient populations and controls for exposure to this cancer-associated virus.

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