Purification of Papillomavirus Structural Polypeptides from Papillomas by Immunoaffinity Chromatography

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SUMMARY

A broadly cross-reactive monoclonal antibody directed against papillomavirus, coupled to immunoaffinity columns, was used to isolate bovine papillomavirus type 1 (BPV-1) and human papillomavirus type 1 (HPV-1) structural polypeptides from homogenates of productively infected cells. One of the polypeptides isolated from bovine fibropapillomas appeared to be the BPV-1 major capsid protein since it had a mol. wt. of 54K and was reactive by Western blots with papillomavirus genus- and BPV-1 type-specific rabbit antibodies as well as monoclonal antibodies cross-reactive with BPV-1/BPV-2 and BPV-1/deer PV. A polypeptide from human plantar warts similarly appeared to be the major capsid component since it also had a mass of 54K to 55K and reacted with papillomavirus genus- and HPV type-specific rabbit antibodies. By using this technique structural viral polypeptides from papillomavirus-induced lesions containing readily detectable viral structural antigens but relatively few virus particles, such as seen with mucosotropic HPVs can now be isolated for mapping of virus-specific epitopes.

INTRODUCTION

Human papillomaviruses (HPV) are associated with a variety of hyperplastic, dysplastic and neoplastic lesions of squamous epithelium (Howley, 1982; Jenson et al., 1984; Orth & Favre, 1985; Rowson & Mahy, 1967). Because HPVs cannot replicate in tissue culture systems permissive for expression of structural viral antigens (Butel, 1972), research has been limited to characterization of virions obtained primarily from cutaneous lesions such as plantar and common warts. Recent advances in molecular biology, however, have allowed cloning of HPV DNA extracted directly from cutaneous and mucosal lesions (Heilman et al., 1980). This led to the discovery of multiple (at least 40) minimally related cutaneotropic and mucosotropic HPVs, most of which are associated with lesions that remain benign. However, some mucosotropic PVs, especially HPV types 16 and 18, are associated with lesions that become malignant (Crum et al., 1984; Gissmann, 1984; Jenson et al., 1986; Lancaster et al., 1986; Orth & Favre, 1986; Pfister, 1983). Since HPVs appear to be associated with up to 85% of severe dysplasias and squamous carcinomas of the anogenital tract and at least some squamous carcinomas of the oral cavity (Loring et al., 1985), there is considerable interest in serotyping mucosotropic HPV infections. The feasibility of such serological studies, however, cannot be determined without the development of an appropriate HPV type-specific antigen detection system.

In the present study, we show that a broadly cross-reactive monoclonal antibody (MAb) directed against PV, derived from mice immunized with detergent-disrupted bovine papillomavirus type-1 (BPV-1) can be used in immunoaffinity columns to purify BPV-1 and HPV-1 major capsid proteins obtained from homogenates of wart tissue. PV structural proteins purified in this manner contain type-specific and/or minimally cross-reactive antigenic determinants as determined by reaction with MAb and polyclonal antibodies in ELISA and Western blots.
METHODS

Animals. Six-week-old female BALB/c mice (Bar Harbor Laboratories, Me., U.S.A.) were primed with pristane before inoculation with hybridomas for enrichment of MAbs in ascitic fluid.

Papillomavirus antibodies. BPV-1 (SDS) antibodies were produced in rabbits by immunization with detergent-disrupted, heat-aggregated purified BPV-1 virions (Jenson et al., 1980, 1982). These polyclonal antibodies are reactive with PV genus-specific and BPV-1 type-specific epitopes. MAbs designated AU1, AU2 and AU4 were produced by hybridomas prepared from BALB/c mice immunized with detergent-disrupted, heat-aggregated purified BPV-1 virions (DPV) (Nakai et al., 1986). AU1 is broadly cross-reactive, AU2 is only reactive with BPV-1 and DPV, and AU4 is cross-reactive with BPV-1 and BPV-2. HPV-1 type-specific antibodies were produced in rabbits by inoculation with intact HPV-1 virions (Jenson et al., 1980, 1982).

MAb purification. Ascitic fluid was harvested from pristane-primed mice inoculated with hybridoma cells and lipid was removed from the fluid by extraction with 1,1,2-trichlorotrifluoroethane (Nakai et al., 1986). MAbs were purified using Affi-Gel Protein A MAPS (Bio-Rad). MAb titres were determined by the ELISA method as previously described (Voller et al., 1976) and immunoglobulins were fractionated by ammonium sulphate precipitation. Precipitates were collected by centrifugation (10000 g, 30 min) and dissolved in 2.5 ml buffer A (0.2 M-NaHCO$_3$, 0.3 M-NaCl, pH 8.0). Ammonium sulphate was removed by buffer exchange through a column of gel. MAbs were coupled by continuous gentle agitation for 16 h at 4 °C. Uncoupled MAb was removed by precipitation. Precipitates were collected by centrifugation (10000 g, 30 min) and dissolved in 2.5 ml buffer A (0.2 M-NaHCO$_3$, 0.3 M-NaCl, pH 8.0). Ammonium sulphate was removed by buffer exchange through a column of PD-10 (Pharmacia) and the protein concentration estimated by $A_{280}$.

Preparation of immunoaffinity column. Affi-Gel 10 (Bio-Rad) was washed with cold H$_2$O on glass fritted funnels. Six ml of moist gel was mixed with 18 ml of partially purified MAb at a concentration of 24 mg protein per ml of gel. MAbs were coupled by continuous gentle agitation for 16 h at 4 °C. Uncoupled MAb was removed by centrifugation and the reactive esters in the gel were blocked by adding 6 ml of 0.1 M-ethanolamine (pH 8.0) and agitating for 1 h at 4 °C. The gel was collected by centrifugation, resuspended in phosphate-buffered saline (PBS, pH 7.4), packed into columns (15 × 50 mm) and equilibrated with PBS.

Isolation of PV structural polypeptides from immunoaffinity columns. Five mg of tissue from a previously characterized BPV-1-induced fibropapilloma (Lancaster & Olson, 1978) or HPV-1-induced human plantar wart tissue was suspended in 5 ml of PBS and homogenized using a Waring blender. The homogenate was brought to 0.24 M-2-mercaptoethanol, 1% SDS and heated for 5 min at 100 °C. The homogenate was diluted to 50 ml with PBS, clarified by centrifugation (10000 g, 30 min, 4 °C) and applied to an immunoaffinity column. The sample was percolated over the column five times. The column was washed with 20 vol. of buffer B (0.5 M-NaCl, 0.25 M-Tris-HCl, 0.2% Triton X-100, pH 7.5) followed by six column vol. of buffer C (0.15 M-NaCl, 0.1% Triton X-100) collected as 4 ml fractions. The column was eluted at low pH with buffer D (0.2 M-acetic acid, 0.15 M-NaCl, 0.1% Triton X-100, pH 2.5). One ml fractions were collected into tubes containing 0.34 ml 1 M-Tris-HCl (pH 9.0) for an adjusted pH of 7.5 to 8.0. Each of the fractions was dialysed against distilled water and protein concentrations were determined by the Bio-Rad protein assay. Immunological reactivity of each fraction with PV broadly-cross-reactive and type-specific antibodies was assayed by the sandwich ELISA method (described below).

Sandwich ELISA. The sandwich ELISA was performed in 96-well plates (Immulon II, Dynatech Laboratories, Alexandria, Va., U.S.A.) coated with ascitic fluid containing MAb in 0.05 M-carbonate, pH 9.0, overnight at 4 °C followed by 1% bovine serum albumin in PBS for 2 h at room temperature. A sample of each column fraction was added to the wells for 1.5 h, washed, incubated with anti-BPV-1(SDS) antibodies or rabbit sera diluted with TBS. The blots were washed three times with PBS and incubated with alkaline phosphatase-conjugated goat (IgG) anti-mouse or anti-rabbit IgG (Bio-Rad) for 1.5 h. The reactions were developed by immersing the membranes in a solution containing 0.1% naphthol AS-BI phosphoric acid, 0.1% fast violet B salt, 0.001 M-MgCl$_2$, 0.1% Triton X-100, pH 9.5, followed by three washes with TBS. To determine the electrophoretic mobility of detectable polypeptides, parallel blots were stained by India ink (Hancock & Tsang, 1983). Polypeptide standards were electrophoresed through 9% polyacrylamide gels containing 0.1% SDS. Polypeptides separated by SDS–PAGE were transferred electrophoretically to nitrocellulose membranes (Towbin et al., 1979). Blots were washed overnight at 4 °C in 0.4% Tween 20, 0.15 M-NaCl, 0.02 M-Tris–HCl pH 7.5 (TBS) and incubated for 1.5 h at room temperature with MAb, polyclonal antibodies or rabbit sera diluted with TBS. The blots were washed three times with PBS and incubated with alkaline phosphatase-conjugated goat (IgG) anti-mouse or anti-rabbit IgG diluted in TBS for 1.5 h at room temperature. The reaction was developed by immersing the membranes in a solution containing 0.1% naphthol AS-BI phosphoric acid, 0.1% fast violet B salt, 0.001 M-MgCl$_2$, 0.1% Triton X-100, pH 9.5, followed by three washes with TBS. To determine the electrophoretic mobility of detectable polypeptides, parallel blots were stained by India ink (Hancock & Tsang, 1983). Mol. wt. were estimated by electrophoresing protein standards in parallel.

RESULTS

MAbs reactive with broadly cross-reactive antigenic determinants (AU1) coupled to immunoaffinity columns efficiently bound immunoreactive material present in PV-induced lesions. The bovine fibropapilloma homogenate contained a total of 2600 µg of soluble protein
Purification of papillomavirus proteins

Fig. 1. Immunoaffinity purification of BPV-1 structural polypeptides using a broadly cross-reactive MAb. Five mg of BPV-1 fibropapilloma homogenate was passed through an immunoaffinity column containing AU1. ELISA values (O) were determined after reaction with anti-BPV-1(SDS). The homogenate contained an ELISA value of 16.5. After percolation through the immunoaffinity column, the total ELISA value of the homogenate dropped to 13.95. An insignificant amount of protein (△) and immunoreactive material was eluted from the loaded column by neutral (A) buffer washes. However, 24.6 μg of total protein with an ELISA value of 4.28 was eluted from the column by washes at low pH (B). This represents an average purification of 27-fold of immunoreactive protein in the eluates over that of the crude homogenate.

Fig. 2. Immunoaffinity purification of HPV-1 structural polypeptides using a broadly cross-reactive MAb. Five mg of HPV-1 plantar wart homogenate was passed through an immunoaffinity column containing AU1. ELISA values (O) were determined by reactivity with anti-BPV-1(SDS). The homogenate contained 1390 μg of soluble protein (△) with an ELISA value of 0.25. Percolation of the homogenate through the immunoaffinity column caused a drop in total ELISA value to 0.1. Although no detectable ELISA immunoreactive material was present in the neutral washes (A), the low pH washes (B) eluted a total ELISA value of 0.19. This represents an average purification of 133-fold over the crude homogenate.

with a total $A_{410}$ in the ELISA test of 16.5. After percolation through the immunoaffinity column, the total $A_{410}$ of the homogenate in the ELISA test dropped to 13.95. Although the yield of the purification seems poor, the column was overloaded and there was only one percolate. (However, the column can be eluted, washed and re-used many times for binding immunoreactive material from multiple percolates of the same homogenate.) Washes with buffers C and D failed to elute a significant amount of either protein or immunoreactive material from the column (Fig. 1). However, the low pH buffer (buffer D) eluted both protein and immunoreactive material. The total protein collected in the buffer D elution was 24.6 μg; the total $A_{410}$ in those fractions was 4.28. This represents an average purification of 27-fold over that of the crude homogenate. The elution, however, was heterogeneous in that the degree of purification for each low pH buffer fraction varied from a peak of 1800-fold to a trough of 10-fold suggesting that a gradient in the pH or salt concentration may allow for finer fractionation of the immunoreactive material eluting from the column.

Similarly, immunoreactive material in an HPV-1-induced plantar wart homogenate could be partially purified using AU1 MAb in an immunoaffinity column (Fig. 2). The original crude homogenate contained 1390 μg of soluble protein and 0.25 total $A_{410}$ in ELISA. Percolation of the homogenate through the immunoaffinity column dropped the total ELISA value to 0.1. Although no detectable ELISA-reactive material was present in the washes, the low pH washes
Fig. 3. Western blot analysis of the BPV-1 structural polypeptides in immunoaffinity column eluates. The immunoreactivity of BPV-1(SDS) with pooled low pH eluate (E) was compared to its immunoreactivity with the crude fibropapilloma homogenate (F) and sucrose gradient-purified BPV-1 virions (B). The reactivity of MAbs AU1, AU2 and AU4 with the polypeptides in E and B are also shown. The major structural component of BPV-1 (B) bands at 54K and is present in the crude homogenate (F) as well as the low pH column eluate (E). The 48K polypeptide is probably a minor structural component of BPV-1 virions. An unreactive -MAb is included as a control.

Fig. 4. Western blot analysis of immunoaffinity column-purified HPV-1-induced plantar wart homogenate. Western blots of protein in the low pH buffer wash from the immunoaffinity column reacted with BPV-1(SDS) and HPV-1 type-specific polyclonal rabbit antibodies (a). Both antisera detected the major capsid protein (55K) and a minor structural polypeptide (60K). Unreactive (-) serum was included as a control.

eluted a total ELISA value of 0.19. This represents an average purification of 133-fold over the crude homogenate. As with the bovine fibropapilloma homogenate, the plantar wart immunoreactive material eluted in a heterogeneous fashion in the low pH washes. The protein concentration for the plantar wart homogenate was lower than that seen for the bovine fibropapilloma because of the relative higher degree of hyperkeratosis which could not be dissected from the lesion. The lower ELISA values were probably due to the decreased number of available epitopes carried by HPV-1 structural polypeptides reactive with anti-BPV-1(SDS) as compared to reactivity of BPV-1 polypeptides with the BPV-1 type- and PV genus-specific antibodies that constitute anti-BPV-1(SDS).

To determine whether the immunoreactive material purified by the immunoabsorbent column represented PV structural components, polypeptides eluting in the low pH washes were electrophoresed in SDS-PAGE gels and tested for immunoreactivity in Western blots. Polypeptides of BPV-1 virions purified after CsCl banding were tested against anti-BPV-1(SDS) (Fig. 3). Three major structural components were detected at 78K, 54K and 48K. Although the homogenate contained numerous reactive bands, the major reactions occurred at 98K, 78K, 54K and 48K. These results suggest that the homogenate contained BPV-1 major structural components. The major components which eluted from the immunoabsorbent column showed the same banding pattern as seen with purified BPV-1 virions in that the 78K, 54K and 48K polypeptides were resolved. However, the 78K was present in much smaller amounts relative to the 54K and 48K species seen in the virion and homogenate preparations. Although the 48K protein could represent a degradation product of the 54K protein, it is consistently resolved from all capsid preparations and, therefore, may represent a distinct polypeptide. MAbs AU1
(broadly cross-reactive), AU2 (BPV-1 and DPV cross-reactive) and AU4 (BPV-1 and BPV-2 cross-reactive) all reacted with the 54K and 48K polypeptides present in purified BPV-1 and those eluted from the immunoabsorbent column. India ink staining patterns (data not shown) of the crude homogenate produced a smear whereas the protein pattern for purified BPV-1 virions and pooled column eluate correlated with the anti-BPV-1 staining pattern. The plantar wart homogenate polypeptides eluting from the immunoabsorbent column reacted with both anti-BPV-1(SDS) and HPV-1 type-specific polyclonal rabbit antibodies (Fig. 4). The major immunoreactive component banded at 55K with slightly less intense staining at 60K.

**DISCUSSION**

BPV-1 and HPV-1 structural polypeptides were purified from wart tissue homogenates passed through immunoaffinity columns coupled with a MAb reactive with PV broadly cross-reactive antigens. Polypeptides corresponding to the major capsid proteins had a mass a 54K to 55K and carried reactive genus- and type-specific and minimally cross-reactive epitopes when examined by Western blots. The polypeptides correspond in size to the mol. wt. of the major capsid proteins of BPV-1 and HPV-1 observed by us (Nakai et al., 1986) and others (Favre et al., 1975; Gissmann et al., 1977; Lancaster & Olson, 1978; Orth et al., 1978; Roseto et al., 1984). Thus, this technique may circumvent the necessity of purifying large quantities of intact virions for immunological studies of native PV structural polypeptides.

PVs are classified into types and subtypes based on species specificity and polynucleotide sequence homology. Virus isolates are considered new types if there is less than 50% nucleic acid homology with existing known types; PV with greater than 50% but less than 100% nucleic acid homology are classified as subtypes (Coggin & zur Hausen, 1977). Little is known, however, of PV serotypes. Type-specific serological reagents only exist for those HPV (eg. HPV-1) which replicate to high enough concentrations in cutaneous warts to be easily purified for immunological studies. On the other hand, mucosotropic PV infections appear to be characterized by the accumulation of intranuclear structural viral protein. The observation of areas of intense nuclear staining in laryngeal papillomas with anti-BPV-1(SDS) with detection of only a few intranuclear virus particles by electron microscopy tends to support this hypothesis (Lancaster & Jenson, 1981). In addition, virus particles have not been purified from condylomas although high concentrations of virus DNA can readily be isolated (Grussendorf-Cohen et al., 1983).

Isolation of excess structural PV polypeptides by immunoaffinity columns could provide a source of naturally occurring mucosotropic HPV structural antigens. Analysis of these proteins could provide insights into processing of mRNA derived from late region open reading frames. Alternatively, the immunoreactive domains of the major capsid protein of HPVs could be mapped. However, the presence of type-specific and cross-reactive domains on the major capsid protein suggests that enzymatic digestion or other alternatives such as the use of synthetic oligopeptides or fusion proteins may be necessary to develop a test to detect PV serotypes.

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**REFERENCES**


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