Delayed-type hypersensitivity response to human papillomavirus type 16 E6 protein in a mouse model

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A mouse model incorporating the epitheliotropic nature of human papillomavirus (HPV) infections has been used to study an immune response to HPV type 16 (HPV-16) E6 protein in vivo. Using a transplantation technique, a novel immortal keratinocyte cell line expressing the E6 protein has been grafted onto syngeneic mice to re-form a differentiated epithelium overlying a granulation tissue bed. By this approach the presentation of viral antigens to the immune system can be modelled in a way analogous to the natural infection. Here we report a delayed-type hypersensitivity (DTH) reaction in grafted mice challenged intradermally with a recombinant vaccinia virus expressing the HPV-16 E6 protein. The specificity of the response was confirmed by the absence of a DTH reaction to challenge with virus expressing either HPV-16 E7 or L1 protein.

More than 65 different types of human papillomavirus (HPV) have been described, classified on the basis of nucleotide homology. At least 20 genotypes are associated with lesions of the genital tract. Benign genital warts (condylomata acuminata of low malignant potential) are most frequently associated with types 6 and 11, whereas types 16 and 18 are found predominantly in anogenital dysplasias and carcinomas.

Our understanding of the immunobiology of papillomaviruses, although limited, is central to any strategy for prophylactic or therapeutic intervention. The evidence from immunosuppressed patients (Alloub et al., 1989; Kent et al., 1987; Sillman et al., 1984; Lutzner et al., 1983; Mullen et al., 1976) and regressing warts (Fierlbeck et al., 1989; Aiba et al., 1986) suggests an important role for the immune system in papillomavirus infections.

Humoral responses to the transforming proteins E6 and E7 in benign genital disease are low (Jenison et al., 1990) in contrast to the levels of antibody response demonstrated by the serum of patients with cervical carcinoma (Muller et al., 1992; Mann et al., 1990; Dillner, 1990; Jochmus-Kudielka et al., 1989). Despite this, doubt still exists as to whether seroconversion is an inevitable consequence of HPV infection, and furthermore no apparent benefit has been attributed to the presence of HPV-16- or -18-specific antibodies in the serum. In contrast to humoral immunity there is good evidence that cell-mediated immune responses are important in the pathogenesis of HPV infections. The spontaneous regression of HPV-associated lesions is accompanied by a pronounced local infiltration of inflammatory cells including cytotoxic T lymphocytes (CTLs), macrophages and natural killer cells (Fierlbeck et al., 1989; Aiba et al., 1986), and a histological appearance characteristic of a delayed-type hypersensitivity (DTH) response.

Recently, an animal model has been described that enables immune responses to HPV-16 early gene proteins within the BALB/c mouse to be studied (McLean et al., 1993). The model incorporates the exclusively epithelial nature of HPV-16 by the presentation of antigen to the animal as a differentiated syngeneic epithelial graft expressing viral protein. Mice primed in this way with HPV-16 E7-expressing cell lines respond with a DTH reaction on subsequent challenge with recombinant E7 protein (McLean et al., 1993; M. A. Chambers, Z. Wei, N. Coleman, A. A. Nash & M. A. Stanley, unpublished results). The reaction is specific to the E7 protein and is dependent on the presence of CD4+ T cells. Using this animal model we have demonstrated a DTH reaction to recombinant E6 protein specifically in mice primed by the grafting of E6-expressing syngeneic keratinocytes.

The materials and methods used in this report have been described previously (McLean et al., 1993), with the following exceptions. BALB/MK cells were obtained from S. A. Aaronson, Laboratory of Cellular and Molecular Biology, National Cancer Institute, Bethesda, Md., U.S.A. and are described in Weissman & Aaronson (1983). They were cultured in keratinocyte serum-free
Fig. 1. Expression of E6 protein by VACE6 early (a) and late (b) in infection. CV-1 cells were infected with 10 p.f.u./cell VACE6 (odd-numbered lanes) or parental virus WR (even-numbered lanes) for (a) 4 h or (b) 12 h. Cells were metabolically labelled with [35S]cysteine from 2 h p.i., then lysed in RIPA buffer and immunoprecipitated as described previously (Stacey et al., 1992). Sera used for immunoprecipitation were: (a) polyclonal anti-E. coli-derived β-galactosidase-E6 fusion protein (lanes 1 and 2); cervical carcinoma patient A (lanes 3 and 4); normal human control serum (lanes 5 and 6); polyclonal anti-vaccinia virus (lanes 7 and 8); (b) cervical carcinoma patient A (lanes 1 and 2); cervical carcinoma patient B (lanes 3 and 4); anti-E6 monoclonal antibody C1P5 (lanes 5 and 6); polyclonal anti-MS2–E6 fusion protein (lanes 7 and 8). M_r markers are shown on the right of each panel (lanes M).

medium (SFM) (Gibco-BRL) supplemented with the bovine pituitary extract and human epidermal growth factor supplied with the medium in a 31 °C humidified atmosphere containing 5% CO₂.

Recombinant vaccinia virus expressing HPV-16 E6 protein was constructed as follows. The HPV-16 E6 open reading frame (ORF) was isolated as a 542 bp EcoO109(112) to Ddel(654) fragment, blunt-ended with the Klenow fragment and BamHI (CGGATCCG) linkers were attached. After digestion with BamHI the fragment was ligated into the BamHI site of vaccinia virus insertion vector p1109, which contains the 7.5K promoter and an in-frame NcoI start codon (M. Mackett, unpublished results). This resulted in amino acids 2 and 3 of the E6 ORF (F and Q) being replaced with two vector-encoded amino acids (D and P). The insertion vector also contained the dominant selectable gpt marker gene. The vector was used to insert the E6 ORF into the thymidine kinase (TK) locus of the WR strain of vaccinia virus, by selection for the gpt marker. The recombinant virus was produced by transfection of CV-1 cells with 5 x 10⁵ cells. Virus was added to 3 ml of SFM containing 4 μg/ml polybrene (hexadimethrine bromide; Sigma). The cells were incubated for 8 h prior to Southern blotting and by testing for the TK− phenotype using BUdR selection (data not shown). Expression of the E6 protein was investigated using the radioimmunoprecipitation assay (RIPA) (Fig. 1). This figure shows that E6 was expressed by VACE6 but not by WR at both early and late times following infection. The E6 protein could be detected by only some antisera raised against Escherichia coli-derived fusion proteins, in line with previous observations (Stacey et al., 1992). This suggests that the dominant epitopes recognized by these antisera are masked or buried in the structure of the E6 protein when it is in its native conformation. Sera from E6-seropositive cervical carcinoma patients readily recognized the vaccinia virus E6 protein in RIPAs, demonstrating the antigenic authenticity of the recombinant protein.

To determine whether mice would mount a DTH response to HPV-16 E6 [as reported by McLean et al. (1993) for HPV-16 E7] two new cell lines were derived by infection of BALB/MK cells with either the recombinant retrovirus LXSN or LXSN16E6 (obtained from C. L. Halbert, Fred Hutchinson Cancer Research Center, Seattle, Wash., U.S.A.; described in Halbert et al., 1991). Infection of cells was carried out in 60 mm dishes seeded with 5 x 10⁵ cells. Virus was added to 3 ml of SFM containing 4 μg/ml polybrene (hexadimethrine bromide; Sigma). The cells were incubated for 8 h prior to
We examined the ability of $10^7$ MKLE6SN cells to form an epithelium when grafted onto syngeneic mice (methodology as in McLean et al., 1993). Seven days after grafting the cells formed an epithelium exhibiting full thickness atypia with very limited differentiation potential. The graft had a highly irregular basal layer and in some levels there was early invasion of the granulation tissue bed (data not shown). This is not a consequence of E6 expression since a similar phenomenon has been seen with the control line MKLXSN, containing no HPV sequences (data not shown).

To assay for DTH reactivity to HPV-16 E6, mice were grafted with $10^7$ MKLE6SN cells and challenged intradermally 7 days later in the left ear with $10^7$ p.f.u. recombinant VACE6 or VACL1. Similarly, three control groups of mice were sham-grafted with 200 μl PBS and subsequently challenged with $10^7$ p.f.u. VACE7, VACL1 or VACE6. A significant ear swelling response ($P < 0.002$, Student’s $t$-test) was seen in the group grafted with $10^7$ MKLE6SN cells and challenged with VACE6 compared with each of the control groups (see Fig. 3). We conclude that HPV-16 E6 protein produced in the epithelium can prime immunocompetent mice in an antigen-specific manner.

The immunogenicity of HPV-16 E6 has been demonstrated before. Serum antibody responses from cervical carcinoma patients have been demonstrated using baculovirus-derived recombinant E6 protein as the target antigen (Stacey et al., 1992) and to specific E6 peptides (Muller et al., 1992). However, for humoral responses it appears that reactivity to HPV-16 E6 is associated with HPV-16-containing cervical cancer but not to HPV-negative cervical cancer or interestingly to HPV-16-containing cervical intraepithelial neoplasia (CIN) (Ghosh et al., 1993). For some time it has been known that the ability to mount a DTH reaction to specific viral or non-specific antigens is a factor in wart immunity (Thivolet et al., 1977). For example, positive skin tests have been reported in patients with regressing warts or with a history of past warts challenged with purified papillomavirus capsid antigen (Viac et al., 1977), in CIN patients on challenge with HPV-16 L1 peptides expressed as recombinant bacterial fusion proteins (Hopfl et al., 1991), whereas contact dermatitis induced by dimeth-chlorobenzene at the site of recalcitrant verrucae provokes their rejection (Greenberg et al., 1973). To date, the bulk of cell-mediated immunity studies on HPV-16 have focused on responses to the E7 protein with a few notable exceptions. Stauss et al. (1992) have demonstrated three murine class I (H-2Kb or D3) binding peptides within HPV-16 E6 capable of stimulating primary CTL responses in vitro, and have underlined the importance of peptide length for CTL induction. Extrapolation to the human situation has proved less successful, however. In a typical study, only one of 29 refeeding with fresh SFM. Clones of infected cells were established by selection in medium containing 200 μg/ml G418 (Sigma). Infection of cells with LXSN resulted in the monoclonal line MKLXSN, containing no HPV sequences. The infection of cells with LXSN16E6 resulted in the monoclonal line MKLE6SN which by RIPA was shown to express the HPV-16 E6 protein (see Fig. 2).

Fig. 2. Immunoprecipitation of HPV-16 radiolabelled E6 protein. Samples were precipitated with rabbit polyclonal E6 antiserum and run on a 17.5% polyacrylamide gel prior to autoradiography. Lane 1, MKLE6SN cells; lane 2, MKLXSN cells; lane 3, CaSki cells (human cervical squamous cell carcinoma cell line containing integrated HPV-16). The positions of size markers and the E6 protein are given.

Fig. 3. DTH response to specific recombinant vaccinia virus challenge. Mice were grafted with or without $10^7$ MKLE6SN cells and challenged 7 days later in the left ear with $10^7$ p.f.u. of the vaccinia virus recombinant shown. The graph shows the difference in thickness between the left and right (unchallenged control) ears measured 24 and 48 h after intradermal inoculation. Each group contained five mice: error bars represent the S.D. of the group. Production of VACE6 is described in the text. VACE7 and VACL1 have been described previously (McLean et al., 1993). A—A, MKLE6SN+VACE6; ■—■, MKLE6SN+VACL1; ○—○, No graft+VACE6; □—□, No graft+VACL1; △—△, No graft+VACE6.
patients with CIN was positive in a lymphocyte proliferation assay using purified HPV-16 E6 protein (Cubie et al., 1989) and only one T cell determinant in HPV-16 E6 (DR7Dw7-restricted) has been defined using synthetic peptides to stimulate peripheral blood mononuclear cells from normal individuals (Strang et al., 1990).

The ability of HPV-16 E6 to act as a tumour rejection antigen has been demonstrated following immunization with recombinant E6 vaccinia virus, in the rat (Meneguzzi et al., 1991), and with cells expressing the E6 protein, in the mouse (Chen et al., 1992). In the latter model, E6-specific, CD8+ CTLs could be generated in vitro from spleen cell populations derived from the E6-immunized mice.

To our knowledge, the data presented here are the first demonstration of an in vivo CD4+ mediated response to the HPV-16 E6 protein in the mouse. Taken together with the demonstration of a DR-restricted determinant in HPV-16 E6 (Strang et al., 1990), this may indicate a role for CD4+ cells in the response to HPV additional to that implied for CD8+ CTLs. In view of these data and those of Chen et al. (1992) the E6 protein of HPV-16 is an attractive candidate as a target antigen in any vaccine strategy. Although the WR-based E6 recombinant vaccinia virus described here is too virulent for use in humans, the model system described here provides an ideal opportunity for testing the antigenicity of poxvirus recombinants based on more attenuated forms of the virus.

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References


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