Delayed-type hypersensitivity response to the human papillomavirus type 16 E7 protein in a mouse model

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To study the immune response to human papillomavirus type 16, a mouse model was developed using a mouse keratinocyte cell line expressing the E7 protein. This line was grafted onto syngeneic mice to form a differentiated epithelium, thus closely mimicking the natural infection.

Introduction

Human papillomaviruses (HPVs) are small DNA viruses that cause a variety of proliferative lesions on cutaneous or mucosal epithelia. They have been classified into more than 60 different types by DNA sequence analysis (de Villiers, 1989). There is now increasing evidence that a subgroup of HPVs, including HPV-6, -11, -16, -18 and -33, is associated with benign and malignant lesions of the genital tract (zur Hausen & Schneider, 1987). Of these, the HPV-16 genome and its products have been identified most frequently in cervical dysplasias and carcinomas (Galloway & McDougall, 1989; Hoover & Fraumen, 1973).

Although rapid progress has been made in the understanding of the molecular biology of HPV-16, this has not been matched by an equal increase in our understanding of the immune response to this virus. There is no doubt that the immune system plays an important role in the pathogenesis of papillomavirus-induced lesions. Infections with HPV are one of the most frequent viral complications in immunosuppressed individuals. For instance, renal allograft recipients have a 10-fold increase in the relative risk of developing cervical neoplasia (Hoover & Fraumen, 1973; Porreco et al., 1975; Shokri-Tabibzaden et al., 1981; Schneider et al., 1982). Regression of flat warts shows many characteristics of a cell-mediated immune response: the lesion is infiltrated by mononuclear cells in a reaction resembling a delayed-type hypersensitivity (DTH) reaction (Tagami et al., 1974; Berman & Winkelman, 1977). Although some correlation has been found between the presence of antibodies to HPV-16 proteins and cervical cancer (Mann et al., 1990; Müller et al., 1990), their biological importance is difficult to judge.

Our understanding of the role of the immune system in HPV infection could be enhanced through a suitable animal model incorporating some of the important viral characteristics. One of the major difficulties in developing such a model has been the lack of a tissue culture system for HPV propagation, resulting in the need for alternative expression systems (McLean et al., 1990; Browne et al., 1988).

HPV is an exclusively intraepithelial pathogen and viral antigens are presented to the immune system via the skin or mucosal surface. Any animal model needs to take account of this requirement to reflect the natural infection as closely as possible. To achieve this, we have derived a mouse keratinocyte cell line expressing the HPV-16 E7 protein. This line was then grafted onto syngeneic mice to form a differentiated epithelium, using a technique originally described by Worst & Fusenig and modified by Hammond (Yeoman et al., 1989; Hammond et al., 1987). We chose to examine the immune response to the E7 protein in the first instance, as this protein has been implicated in transformation and is found in all human cell lines containing HPV-16 DNA. In our model, we examined DTH responses to the HPV-16-containing line because these reactions were shown to be implicated in wart regression, and are exclusively associated with muco-cutaneous surfaces. A vaccinia virus construct expressing the HPV-16 E7 protein was used to induce the DTH reaction.

Methods

Mice. BALB/c mice, aged 6 to 8 weeks, were bought from OLAC.

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Cells and viruses. Primary keratinocyte cells were obtained from the tails of BALB/c adult mice using a previously described trypsin flotation procedure (Yeomans et al., 1989). Epidermal cells (primary or transfected) were plated onto tissue culture dishes in the presence of 2×10^9 lethally irradiated 3T3 feeder cells per 75 cm^2 plate and grown in Glasgow modified Eagle’s medium (GMEM) supplemented with 10% foetal calf serum (FCS), 0.1 µg/ml hydrocortisone, 4×10^-9 M cholera toxin and 100 µg/ml kanamycin sulphate (Sigma). CVI cells and feeder 3T3 cells were maintained in GMEM/10% FCS at 37 °C in a humidified atmosphere containing 5% CO₂. Mouse keratinocytes were grown at 31 °C/5% CO₂. The virus strain used for the production of recombinant vaccinia virus was Western Reserve (WR). The construction of the recombinant vaccinia virus expressing the L1 open reading frame (ORF) (L1 Vaccinia) has been described previously (Browne et al., 1988). Virus strains were grown in BHK-21 cells and titrated on CV1 cells.

Transfection of keratinocytes. Subconfluent primary cultures of BALB/c mouse epidermal keratinocytes (NEKs) were transfected with 5 µg of plasmid DNA (pJ4Ω16, see Fig. 1) and 5 µg of the selectable marker pSVneo using the calcium phosphate coprecipitation technique (Wigler et al., 1979). Plasmid pJ4Ω16 and pSV2neo DNA were mixed with 200 µl of TE buffer (10 mM-Tris-HCl pH 7.9, 1 mM-EDTA) and 20 µl of a 2.5 M solution of CaCl₂ was slowly added. After addition of 200 µl 2 × HBS (280 mM-NaCl, 50 mM-HEPES, 1.5 mM-Na₂HPO₄, pH 7.05, with NaOH) the mixture was vortexed and left for 20 min to form a precipitate. This was added to a subconfluent grown culture dish and incubated overnight at 31 °C. Cells were shocked with glycerol (20% in PBS), washed in PBS and left for 1 day in fresh medium. After passage onto fresh 3T3 feeder layers at a density of 1:2, cells were selected with G418 at 100 µg/ml for 4 days. Colonies appeared after 2 to 3 weeks and were expanded by serial passage.

Grafting. Two weeks prior to grafting, a glass coverslip was inserted under the dorsal skin of BALB/c mice to form a granulation tissue bed. A transplantation chamber (Renner GmBH) was inserted after removal of the coverslip and cells were injected into the chamber at a density of 2×10^5, with NaOH) the mixture was vortexed and left for 20 min to form a precipitate. This was added to a subconfluent grown culture dish and incubated overnight at 31 °C. Cells were shocked with glycerol (20% in PBS), washed in PBS and left for 1 day in fresh medium. After passage onto fresh 3T3 feeder layers at a density of 1:2, cells were selected with G418 at 100 µg/ml for 4 days. Colonies appeared after 2 to 3 weeks and were expanded by serial passage.

Preparation of DNA and PCR analysis. Cells were lysed in 10 mM-Tris-HCl pH 7.5, 10 mM-EDTA, 0.6% SDS and 0.25 mg/ml proteinase K overnight at 50 °C, and extracted twice with phenol, twice with a mixture of phenol–chloroform and twice with chloroform. DNA was precipitated with two volumes of ethanol, spooled and air-dried. The purified DNA was dissolved overnight at 4 °C in TE buffer (pH 7.5). PCR reaction consisted of 30 cycles: 2 min denaturation at 92 °C, 2 min annealing at 50 °C and 10 min primer extension at 70 °C. The primers were taken from the upstream regulatory region (URR) of the HPV-16 genome and had the following sequences: ACCGGCGCATGATTGCCCACGACAGTCTGTGGCAAA (kind gift of Dr A. Storey, ICRF TVG, Cambridge, U.K.). Each PCR reaction was subjected to three rounds of plaque purification and dot blot analysis. Western blot analysis using diaminobenzidine (DAB). Analysis of HPV proteins expressed by vaccinia virus recombinants was essentially as described previously (McLean et al., 1990). BHK-21 cell monolayers were infected with recombinant vaccinia virus for 18 h and lysed in sample buffer (24 mM-Tris–HCl pH 6.8, 100 mM-DDT, 2% SDS, 20% glycerol, 0.02% bromophenol blue). After sonication, samples were denatured at 100 °C for 3 min and subjected to SDS-PAGE. Proteins were transferred to nitrocellulose by electroblotting and filters were blocked in 5% dried milk powder, 1% FCS in PBS before reaction with the primary antibody at an appropriate dilution in PBS/1% FCS for 30 min at room temperature. The filters were washed three times in PBS 1% NP40 and incubated with biotinylated anti-IgG1 (1:200 dilution, Amersham) for 1 h at room temperature. After three washes in PBS, streptavidin peroxidase (Amersham) was added at a dilution of 1:350 in PBS for 30 min, washed three times in PBS and bands were visualized with a DAB solution (0.05 M-Tris–HCl pH 7.6, 0.5 mg/ml DAB, 0.25 µl/ml 30%H₂O₂).

Production of recombinant vaccinia virus. This was performed essentially as described by Browne et al. (1988). Briefly, confluent monolayers of CV1 cells were infected with wild-type WR vaccinia virus at 0.5 p.f.u./cell for 2 h at 37 °C. Vector pJ57 DNA was precipitated with calcium phosphate and added to the cells for 30 min at room temperature. The inoculum was replaced with GMEM/10% FCS and cells were incubated at 37 °C/5% CO₂ for 2 days, after which they were scraped, frozen, sonicated and used to infect TK-143 cell monolayers at various dilutions under 2% agarose, 5% FCS and 25 µg/ml bromodeoxyuridine in GMEM. Dishes were incubated at 37 °C for 2 days and plaques were picked after visualization with 0.005% neutral red. These plaques were used to infect TK-143 cells for 2 days, after which constructs containing the required gene were identified by dot blot hybridization. Positive virus constructs were subjected to three rounds of plaque purification and dot blot hybridization.

Injection of mice with virus and measurement of ear swelling. Vaccinia virus recombinants were inoculated at a dose of 10^6 p.f.u. in 25 µl GMEM into the pinna of the left ear of anaesthetized mice (Nash et al., 1980). The ear thickness was measured 24 and 48 h afterwards using a Mitutoyo engineer’s micrometer. The right, uninoculated ear was measured as a control. There were five mice in each experimental group, in every experiment.

In vivo depletion of T cells. The antibodies used for in vivo depletion experiments were as described by Cobbold et al. (1984): antibody YTS191 depletes the mouse of CD4⁺ cells, and YTS169 reacts with the CD8⁺ cells. Antibodies were purified by ammonium sulphate saturation and subsequent dialysis against PBS. The final concentration for both antibodies was approximately 10 mg/ml. Injection of antibodies was via the intravenous route, 0.1 ml per mouse, on days -7 and -4 before grafting (i.e. 1 mg antibody per administration). A third injection on day -1 was given intraperitoneally.

Results

Transfection of HPV-16 into mouse keratinocyte lines

The exclusively epithelial nature of HPV-16 makes it desirable to express viral antigens in keratinocytes. Since viral particles cannot be used to infect cells, we have used
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(a) Structure of plasmid pJ4Ω16. Dark blocks represent genes present in the plasmid. By courtesy of Dr A. Storey (Storey et al., 1988). (b) Analysis of NEK-16 DNA. Electrophoresis on 1% agarose gel of DNA obtained after PCR amplification using primers from the HPV-16 URR region. Lane 1, size markers; lane 2, plasmid pJ4Ω16; lane 3, normal keratinocytes; lane 4, cell line NEK-16. (c) Presence of E7 protein. Western blot analysis of cell lines NEK-16 (lane 1) and NEK ras 8 (lane 2) using enhanced chemiluminescence. Filters were reacted with a mixture of antibodies Camvir 3 and Camvir 4 at a 1:100 dilution, washed three times in blocking buffer and incubated with rat-absorbed biotinylated anti-mouse IgG. After three further washes in PBS and incubation with streptavidin peroxidase, bands were visualized using the reaction mixture and film provided in the Amersham kit. Mr markers are shown on the right.

a plasmid containing the entire early and part of the late region of HPV-16 (pJ4Ω16, see Fig. 1a) to transfect primary BALB/c mouse keratinocytes. Transfection was carried out using the calcium phosphate precipitation method. Colonies were apparent at approximately 3 weeks after G418 selection. These were cloned and amplified by serial passage for further analysis.

The presence of HPV-16 DNA in one of the transfected lines (NEK-16) was established by Southern blot hybridization (results not shown) and PCR analysis (Fig. 1b). The primers used in the PCR reaction were taken from the URR region of the HPV-16 genome. DNA taken from the transfected cell line NEK-16 or from two different HPV-16-containing plasmids gave rise to a 900 bp band on agarose gels after being subjected to amplification by PCR, whereas DNA from primary mouse keratinocytes, used as a negative control, did not show this band, confirming that the NEK-16 line contains HPV-16 DNA.

The expression of the HPV-16 E7 protein in NEK-16 cells was analysed by Western blotting. The monoclonal antibodies (MAbs) Camvir 3 and 4 used in this reaction were shown to be directed against the HPV-16 E7 protein as expressed by a vaccinia virus recombinant or in CaSki cells (unpublished results). It is apparent from Fig. 1(c) that both antibodies recognized a protein with an Mr of 21K in NEK-16 cells, and this is absent from a similarly derived BALB/c keratinocyte line expressing the ras oncogene. It was necessary to use the very sensitive method of enhanced chemiluminescence to visualize the E7 protein band.

In vivo formation of epithelium by NEK-16 cells

We examined the ability of NEK-16 cells to form a differentiated epithelium when grafted onto syngeneic mice, using the technique described in Methods. The epithelium shown in Fig. 2 is produced 8 days after grafting 10⁷ cells into the chamber. Although the graft retains a basal layer, this is rather disorganized. The epithelium retains an organized pattern of differentiation with signs of stratification, but nucleated cells can be detected in the upper suprabasal layers.

The graft is rejected at about 16 days after grafting, but the rejection process is accelerated when the mice are challenged with vaccinia virus to elicit a DTH response (see below) and rejection takes place at 11 days post-grafting.
**Construction and analysis of a vaccinia virus recombinant expressing the HPV-16 E7 ORF**

The structure of the plasmid containing the E7 ORF is shown in Fig. 3(a). The E7 ORF was taken from pBR-HPV-16 (kind gift from Professor H. zur Hausen) using HindIII and PvuII digestion. This was placed under the control of the vaccinia virus p4b promoter by ligation into pRK19 (Kent, 1988) which had previously been digested with SmaI and end-dephosphorylated. Correct insertion and orientation were verified using appropriate digestion enzymes. This plasmid, designated pJS7, was used to transfect wild-type vaccinia virus-infected cells to produce an HPV-16 E7 vaccinia virus recombinant (E7 Vaccinia). The DNA of E7 Vaccinia was analysed by Southern blot hybridization and shown to contain the E7 ORF (results not shown). Expression of the E7 protein in recombinant virus-infected cells was confirmed by Western blotting with antibody Camvir 3 (Fig. 3 b). The lysate from E7 Vaccinia-infected cells contained a band with an M_r of 20K, which was absent from the L1 Vaccinia-infected control cells.

**DTH response to NEK-16**

BALB/c mice were sensitized by grafting 10^7 NEK-16 cells per mouse in a manner designed to form a differentiated epithelium. Eight days after grafting, mice were challenged in the left ear pinna by an intradermal injection of 10^7 p.f.u. E7 Vaccinia or L1 Vaccinia. A set of non-grafted mice were injected with the same inoculum in each experiment as a control. Ear thickness was measured at 24, 48 and sometimes 72 h after infection. A markedly increased ear swelling was observed in NEK-16-grafted mice challenged with E7 Vaccinia (Fig. 4a). To confirm further the specificity of this response to the HPV-16 E7 protein, a second experiment was performed in which mice were grafted with 10^7 primary BALB/c keratinocytes containing no HPV-16 DNA (see Fig. 4b). When these mice were challenged with E7 Vaccinia or L1 Vaccinia, the ear thickness did not differ from that observed in control, non-grafted mice, and there was no difference between groups challenged with E7 Vaccinia or L1 Vaccinia. As expected, the mice grafted with NEK-16 cells and challenged with E7 Vaccinia gave an increased ear swelling response.

The importance of the route of sensitization was investigated by injecting mice subcutaneously (s.c.) or intraperitoneally (i.p.) with 10^7 NEK-16 cells (Fig. 4c). Increased ear swelling was observed in s.c. sensitized mice when challenged with E7 Vaccinia, but mice immunized via the i.p. route gave the same response in both L1 Vaccinia- and E7 Vaccinia-injected groups. The ear swelling in the E7 Vaccinia-challenged, s.c. sensitized mice was less vigorous than that observed in the grafted animals.
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Fig. 4. DTH response to E7 Vaccinia in NEK-16 grafted mice. (a) Mice grafted with NEK-16 cells. Groups of five mice were challenged at day 8 post-grafting with 10^7 p.f.u. E7 Vaccinia (E7VAC) or TK^- vaccinia virus (TK^-VAC) by intradermal injection in the left ear pinna. A set of non-grafted control animals were injected with the same virus constructs. Ear thickness was measured at 24, 48 and occasionally 72 h after injection and expressed as the difference (μm) between right and left ears. Error bars represent the standard deviation per group of five mice. (b) Specificity of DTH response for NEK-16 grafted mice. Mice were grafted with 10^7 NEK-16 cells or with 10^7 normal keratinocytes (NEK), and challenged with E7 Vaccinia or L1 Vaccinia as in (a). Non-grafted control mice were also included. (c) Sensitization by i.p. or s.c. immunization. Mice were injected with 10^7 NEK-16 cells either s.c. or i.p.. Two weeks later animals were challenged with E7 Vaccinia or L1 Vaccinia as in (a). (d) In vivo depletion of T cell subsets. Mice were depleted of CD4^+ or CD8^+ cells by administering antibodies YTS169 and YTS191, either separately or combined, by intravenous injection on days -7 and -4 prior to grafting. A third injection on day -1 was given i.p. Mice were grafted with 10^7 NEK-16 cells on day 0 and challenged with E7 Vaccinia or L1 Vaccinia on day 7. A non-depleted set of mice was included, as well as a control set of non-grafted mice. Ear thickness was measured as for (a).

To establish the subset of T cells responsible for the observed DTH reaction, mice were depleted of CD4^+ or CD8^+ cells using antibodies YTS191 and YTS169. These were shown to be effective for in vivo T cell depletion when administered intravenously (Thois et al., 1989). Mice depleted of CD8^+ cells before grafting with NEK-16 gave an ear swelling response similar to non-depleted animals when challenged with E7 Vaccinia. CD4^+-depleted mice on the other hand showed no increased ear thickness compared with double-depleted (CD4^-, CD8^-) mice following E7 Vaccinia challenge (Fig. 4d). The response of CD4^+-depleted mice was similar to that observed in non-grafted or L1 vaccinia virus-challenged individuals, showing that the observed DTH response is mediated by a CD4^+ subset of T cells.

Discussion

In this report we describe a mouse model system for the study of immune responses to papillomavirus antigens. Through the use of a non-tumorigenic mouse keratinocyte cell line containing most of the HPV-16 genome and a transplantation technique that permits the reformation of a differentiated epithelium in vivo it is possible to present HPV-16 antigens to the immune system in a manner closely resembling the natural
infection. Using this approach we have demonstrated a cell-mediated immune response specifically directed against the E7 protein of HPV-16.

The E7 ORF is consistently expressed in HPV-16-containing cell lines and in carcinomas (Smotkin & Wettstein, 1986; Matlashewski et al., 1987; Crook et al., 1990). The E7 protein has the properties of an oncoprotein and has been shown to be essential both for transformation and for the maintenance of the transformed phenotype in a variety of cell systems. A correlation has been shown between the presence of anti-HPV-16 E7 serum antibodies and cervical cancer which suggests that E7 is able to act as a target antigen in the human immune system (Jochmus-Kudielka et al., 1989; Müller et al., 1990). Evidence that E7 can act as a target for a cell-mediated immune response by CD8+ T cells comes from experimental studies using a mouse model (Chen et al., 1991) which showed that mouse melanoma cells transfected with HPV-16 DNA sequences when injected subcutaneously or intraperitoneally could induce a tumour rejection response and that the target for this response appeared to be the E7 protein.

Papillomaviruses are however strictly intraepithelial pathogens. The target cell for infection is the keratinocyte; early viral gene expression may be dependent upon keratinocyte-specific factors (Cripe et al., 1987) and vegetative viral growth is absolutely dependent upon the differentiation programme of the keratinocyte (Sterling et al., 1990). Therefore in the natural infection viral antigen is expressed in keratinocytes and recognized by mucocutaneous immune cell populations. There is increasing evidence that these intraepithelial populations are dominated by lymphocyte subsets that differ significantly from those in lymphoid organs. Thus, in the mouse, γδ T cell populations with distinct T cell repertoires and homing properties have been identified and diversified populations are found in lymphoid organs (Elliott et al., 1988; Takagaki et al., 1989a; Lacey et al., 1989) and intestinal epithelia (Asarnov et al., 1989; Takagaki et al., 1989b). However, specific γδ subsets identified by their use of different Vγ and Vδ TCR genes home to murine skin (Asarnov et al., 1989; Takagaki et al., 1989b) and a different homogeneous subset homes to mucosal epithelium of the tongue and vagina (Itohara et al., 1989). Recent studies have identified, again in the mouse, a skin homing subset of γδ memory T cells (Picket et al., 1991; Shimizu et al., 1991) and these and other data (Mackay et al., 1990) support the notion that there exist several distinct migration streams for T cells and that immunosurveillance at skin and mucosal surfaces is mediated by memory T cells with specific migration pathways (Mackay, 1990). Furthermore there are considerable data to show that keratinocytes can act as effector cells after immune activation synthesizing important regulatory cytokines (Luger, 1989). These observations indicate that it may be important in any animal model to mimic as closely as possible the natural infection with respect to both the infected cell and the route of antigen presentation and processing in order to obtain a clearer understanding of the immune response to HPV.

The animal model which we describe in this study fulfils these requirements and we have used this system to examine the host response to the HPV-16 E7 gene product when the epithelial route of antigen presentation and processing is employed. The immortalized keratinocyte cell line NEK-16 is non-tumorigenic and these cells when grafted onto immunocompetent recipients form a stratified but atypical dysplastic squamous epithelium. NEK-16 cells contain between one and 10 copies of a 6-6 kb fragment of the HPV-16 genome containing the entire early and part of the late region of the genome. These cells were shown to express the E7 protein by Western blotting although the level of expression was low and the protein could be visualized only by the highly sensitive technique of enhanced chemiluminescence. A DTH response was induced in mice grafted with NEK-16 cells and subsequently challenged with E7 Vaccinia. No ear swelling response could be shown when the mice were challenged with L1 Vaccinia, nor could the mice be sensitized by cells that did not express E7. This response was mediated, as might be predicted, by CD4+ cells and would appear to be directed specifically against the E7 protein.

The induction of a DTH response by a transformed but non-malignant HPV-16-expressing keratinocyte line is of considerable interest since there is evidence that such a response is involved in the spontaneous regression of cutaneous flat warts in man. Immunohistological studies reveal that regression of these warts is accompanied by an intense mononuclear dermal infiltrate of CD4+ lymphocytes and HLA-DR+ dendritic cells (Aiba et al., 1986; Chardonnes et al., 1985).

The response is directed against the basal keratinocytes, implying that antigens expressed in basal cells are the primary targets; these are most likely to be early viral proteins such as E7. The animal model described in the present study provides an opportunity to analyse the detailed mechanisms involved in this response and may present a novel approach for immunotherapeutic vaccines in HPV-related disease.

References


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