T cell responses to the human papillomavirus type 16 E7 protein in mice of different haplotypes

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The response of murine T cells to the E7 molecule of human papillomavirus type 16 (HPV-16) was studied using eight different mouse strains of six distinct H-2 haplotypes. HPV-16 E7 protein was prepared as a fusion protein with glutathione-S-transferase, purified by affinity chromatography and used for immunization. Cells from the lymph nodes were cultured with whole fusion protein, glutathione-S-transferase or HPV-16 E7 protein synthetic peptides. All the mouse strains tested, with the exception of BALB/c, recognized the E7 molecule, as evidenced by a proliferative response to at least two of the peptides. The profile of responses to peptides varied between and within a strain, but five distinct immunodominant regions could be identified. These regions were defined on the basis of a reaction to one or more peptides in a given part of the E7 molecule by at least four strains. The five regions were encompassed by amino acid residues 1 to 9, 17 to 32, 42 to 59, 62 to 77 and 87 to 98. The findings suggest that in an outbred population, such as man, the E7 molecule of HPV-16 would be recognized by a large proportion of the population. However, the poor response of two mouse strains [B10.RIII (71NS) and BALB/c] could also have a corollary in man.

Certain human papillomavirus (HPV) types have been implicated in the aetiology of benign and malignant lesions of the ano-genital region. We are interested in identifying immune responses to one of the more important HPV types, namely HPV-16. Little is known about the immunobiology of HPV-16 because the virus cannot easily be grown in vitro or in vivo, and few virions can be isolated from genital lesions. To circumvent these problems, both early and late regions of the HPV-16 genome have been expressed as bacterial fusion proteins (Jenison et al., 1989; Jochemus-Kudielka et al., 1989).

There is limited information on T cell responses to HPV-16 (Strang et al., 1990; Davies et al., 1990), although two groups have recently reported the response of mice to the HPV-16 E7 protein (Comerford et al., 1991; Tindle et al., 1991). The E7 molecule is important because of its proposed involvement in cellular transformation in tissue culture (Matlashewski et al., 1987; Phelps et al., 1988) and because its gene is found integrated into the DNA of a high proportion of carcinomas in which HPV DNA is present (Dürst et al., 1983; Gissmann et al., 1983, 1984). The E7 protein has been considered as a possible candidate for use in vaccination and an immune response to HPV-16 E7 protein has been demonstrated to cause regression of a syngeneic tumour expressing the protein in mice (Chen et al., 1991; Meneguzzi et al., 1991). In this study we have used HPV-16 E7 expressed as a fusion protein with glutathione-S-transferase (GST) together with a series of overlapping synthetic peptides to investigate the immunogenicity of the HPV-16 E7 protein in different mouse strains, and to determine which parts of the E7 molecule may constitute putative T helper cell epitopes.

The GST–E7 fusion protein was induced in Escherichia coli transformed with the plasmid pGEX-2T.HPV16 E7 (Smith & Johnson, 1988; Comerford, 1991), and a crude isolate in the form of insoluble inclusion bodies was prepared according to the method of Marston (1986). This material was solubilized in 8 M-urea and further purified by affinity chromatography using reduced glutathione attached to agarose beads (sulphur-linked, G4510; Sigma). PBS (10 ×) was added to the solubilized GST–E7 protein to give 1 × PBS (16 mM-Na2HPO4, 4 mM-NaH2PO4 and 150 mM-NaCl pH 7·3), and Triton X-100 was then added (1% v/v). The protein solution was passed down a 2 ml column (gel bed volume) and unbound material was collected. After washing with 100 ml PBS, 1% Triton X-100, a stepwise elution of GST–E7 protein was performed by the addition of six 2 ml aliquots of elution buffer to the
column (50 mM-Tris–HCl, 5 mM-reduced glutathione in PBS pH 7.5) and 1 ml fractions were collected. Samples of purified GST–E7 protein were analysed by SDS–PAGE in a mini-gel system (Pharmacia; 3% stacking gel above a 13% resolving gel) and run at 20 mA for 1 to 2 h according to the method of Laemmli (1970). Western blots were also performed on a duplicate SDS–polyacrylamide gel using a Bio-Rad electrophoretic transfer apparatus. The nitrocellulose filters were blocked in PBS containing 10% milk protein, washed thoroughly with distilled water and incubated at room temperature with a mouse anti-HPV-16 E7 monoclonal antibody (CAMVIR 3, kindly supplied by Dr A. Minson, Department of Virology, Cambridge University, U.K.). After 4 h the blots were washed with PBS, 0.05% Tween-20 and the presence of bound antibody was detected by a 2 h incubation with 2 kBq per gel of 125I-labelled goat anti-mouse antibody (specific activity 370 MBq/mg, laboratory-prepared). Autoradiography was performed using X-OMATS film (XSI, Kodak) for 24 h.

The protein eluted from the affinity column and run under reducing conditions in SDS–PAGE migrated in a major band of Mr, 45.5K (Fig. 1a, lane 2), which represented >95% of the eluted protein. The Mr of the band was slightly greater than that predicted for GST–E7 protein (39.5K), but the presence of HPV-16 E7 protein was confirmed by Western blot analysis (Fig. 1b, lane 1). To produce purified GST and HPV-16 E7 protein, the purified fusion protein bound to the affinity column was cleaved with thrombin (T-7009, Sigma; 100 units/ml) and the cleaved material was collected. The protein remaining bound to the column was then eluted with reduced glutathione (5 mM). This was shown by SDS–PAGE to have an Mr of 28K (Fig. 1a, lane 3), as predicted for GST, and did not bind anti-E7 antibody on a Western blot (Fig. 1b, lane 2). The cleaved unbound material ran on SDS–PAGE with an Mr of 22K (Fig. 1a, lane 4), approximately twice the Mr, predicted from the amino acid sequence of the HPV-16 E7 protein, and suggested that under reducing conditions cleaved E7 protein is in dimeric form. This material clearly contained the HPV-16 E7 protein (Fig. 1b, lane 3), as determined by Western blotting.

To investigate murine T cell responses to HPV-16 E7 protein, mice representing a variety of independent H-2 strains B10.S, B10.D2, B10.G, B10.BR and B10.RIII (71NS) were bred by the Biological Services Division, UMDS, London, U.K. Groups of two or three mice were immunized subcutaneously in the hind footpads, and in some experiments the base of the tail, with 20 to 100 µg purified GST–E7 fusion protein emulsified in incomplete (IFA; 0639-59, Difco) or complete Freund's adjuvant (CFA; 3113-60, Difco). One or two weeks after immunization, cells from the draining lymph nodes were cultured in triplicate for 3 days with GST–E7, GST or synthetic peptides in flat-bottomed microtitre plates (2.5 × 10⁵ cells/well) in a humidified 5% CO₂ atmosphere in air (Rayfield et al., 1989). The peptides, which encompassed the whole HPV-16 E7 protein (termed 932 to 950), were synthesized by T-boc chemistry (Houghten, 1985) as 20-mers overlapping by 15 residues, except for the last three peptides which were 19, 14 and nine residues in length, respectively. The culture medium was RPMI 1640 (Gibco) buffered with bicarbonate and HEPES (10 mM), and supplemented with 0.5% mouse serum from thioglycollate-treated CD1 mice (Mullbacher et al., 1985). [Methyl-3H]thymidine (TRA 120, Amersham; 9-25 kBq/well) was added for the final 6 or 18 h of culture, and incorporation was assessed by counting on a liquid scintillation β-spectrometer (LKB).

The data from four separate mouse strains are shown in Fig. 2. All the strains responded strongly to the GST–E7 protein in the range 1 to 20 µg/ml in a dose-dependent manner; for clarity, a single dose is shown in Fig. 2. Positive responses to individual HPV-16 E7 peptides [stimulation index (SI) >2.5, Δd.p.m. >1250] were obtained at a concentration of 10 to 20 µg/ml, but the magnitude of the response was much lower. B10.S (H-2b; Fig. 2a) recognized eight of 19 peptides, peptide 944 (residues 60 to 79, see Table 1) evoking the strongest proliferation response (SI, 9-4, Δd.p.m. 4124). A positive response was also obtained with peptide 943 (residues 55
Fig. 2. Proliferative response of mouse lymph node cells from strains B10.S (a), B10.D2 (b), B10.BR (c) and CBA (d) to HPV-16 E7 proteins and peptides. Groups of three animals were immunized with 10 μg (B10.BR and CBA) or 20 μg (B10.S and B10.D2) affinity-purified GST–E7 protein in IFA and lymph node cells were cultured 12 to 14 days later. Results show mean d.p.m. for triplicate cultures ± standard deviation. The dotted lines represent an SI of 2.5. The concentration of GST–E7 protein was 2.5 μg/ml (a and b) or 1.25 μg/ml (c and d), and the peptides were at 10 μg/ml. Mock-immunized mice gave mean values of 654 ± 210 d.p.m. (a and b) and 5710 ± 345 d.p.m. (c and d) at equivalent GST–E7 protein concentrations.

to 74), but not 942 (residues 50 to 69) or 945 (residues 65 to 84), suggesting that the critical amino acids were between residues 64 and 70. B10.S cells also recognized peptide 932 (residues 1 to 20), but not 933 (residues 5 to 24), implicating the first four amino acids as critical residues. Reactivity with peptides 949 and 950 suggested
Fig. 3. Proliferative response of B10 lymph node cells to HPV-16 E7 proteins and peptides. Groups of three mice were immunized with 20 μg (a) or 100 μg (b) of affinity-purified GST–E7 protein or 100 μg GST–E7 inclusion bodies (c), in IFA (a and c) or CFA (b), and lymph node cells were cultured 11 to 12 days later. Results show mean d.p.m. ± standard deviation for triplicate cultures. The dotted lines represent an SI of 2.5. The concentration of GST–E7 and GST proteins was 2-5 μg/ml, and the peptides were at 10 μg/ml. ND, Not done (a).

Table 1. Summary of responses to HPV-16 E7 synthetic peptides after immunization with GST–E7 protein

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Residues</th>
<th>Strains responding</th>
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<tbody>
<tr>
<td>933</td>
<td>5-24</td>
<td>CBA</td>
</tr>
<tr>
<td>934</td>
<td>10-29</td>
<td>CBA, B10.RIII (71NS)</td>
</tr>
<tr>
<td>935</td>
<td>15-34</td>
<td>B10.S, B10.RIII (71NS), B10.BR</td>
</tr>
<tr>
<td>937</td>
<td>25-44</td>
<td>–</td>
</tr>
<tr>
<td>938</td>
<td>30-49</td>
<td>–</td>
</tr>
<tr>
<td>939</td>
<td>35-54</td>
<td>CBA, B10, B10.BR</td>
</tr>
<tr>
<td>940</td>
<td>40-59</td>
<td>CBA, B10, B10.S</td>
</tr>
<tr>
<td>941</td>
<td>45-64</td>
<td>B10</td>
</tr>
<tr>
<td>942</td>
<td>50-69</td>
<td>–</td>
</tr>
<tr>
<td>943</td>
<td>55-74</td>
<td>B10.S</td>
</tr>
<tr>
<td>944</td>
<td>60-79</td>
<td>CBA, B10, B10.S, B10.G</td>
</tr>
<tr>
<td>945</td>
<td>65-84</td>
<td>B10</td>
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<tr>
<td>946</td>
<td>70-89</td>
<td>CBA</td>
</tr>
<tr>
<td>947</td>
<td>75-94</td>
<td>B10.S</td>
</tr>
<tr>
<td>948</td>
<td>80-98</td>
<td>CBA, B10, B10.D2</td>
</tr>
<tr>
<td>950</td>
<td>90-98</td>
<td>CBA, B10, B10.S</td>
</tr>
</tbody>
</table>

* The criteria for a positive response were SI > 2.5, Δd.p.m. > 1250. Each strain was tested in three separate experiments.

To determine how reproducible the response to an individual peptide was, each strain was tested in a minimum of three separate experiments. The data obtained with B10 (H-2k) animals are shown in Fig. 3. In the first experiment (a), B10 mice immunized with affinity-purified GST–E7 protein recognized seven peptides. In the second experiment (b), five peptides elicited a significant level of proliferation. Only two of the peptides were stimulatory in both experiments (944 and 949), nevertheless the profile of the response to the peptides, with the exception of peptide 936, was similar. In the third experiment (c), the mice were immunized with an inclusion body preparation of GST–E7 protein instead of affinity-purified material; only a single peptide (940) was positive. A comparable variation in the response to individual peptides was found in all the mouse strains tested with the exception of BALB/c. The latter failed to recognize any peptide in six separate experiments, despite clear priming to the fusion protein and GST alone (data not shown).

Table 1 summarizes the overall responses of HPV-16 E7 peptides in the different strains of mice immunized with GST–E7 protein. Although each strain gave a different response profile to individual peptides, five distinct immunodominant regions could be identified. A region was defined on the basis of a reaction to one or more peptides in a given part of the HPV-16 E7 molecule by four or more strains. On the assumption that the proliferative T cell epitopes would span nine amino acids.
congenic strains, representing four distinct H-2 haplotypes (k, b, d and s). A minor determinant was found on the basis of a response to two peptides covering residues 12 to 29 and 18 to 37. They also demonstrated that B10. A (2R) mice (K^k 1-A^k 1-E^k D^k) recognize the major epitope between residues 48 and 54 following immunization with an MS2 fusion protein. A GST–E7 fusion protein used in conjunction with a Protein A–HPV-16 E7 fusion protein in CBA mice provided evidence for four distinct H-2 haplotypes, but this requires further investigation. There is good agreement between the regions defined here and the epitopes identified by Comerford et al. (1991). Only the first epitope (residues 1 to 9) represents a previously silent region because CBA mice do not appear to recognize these residues.

Our results show that seven of the eight mouse strains tested respond to HPV-16 E7 peptides after immunization with a purified GST–E7 fusion protein. Two recent reports have identified T cell determinants on the HPV-16 E7 protein. Tindle et al. (1991) identified a major T cell epitope between amino acids 48 and 54 (DRAHYNI) by immunizing mice with synthetic peptides representing the entire HPV-16 E7 protein. They studied 11 different strains, including recombinant and congenic strains, representing four distinct H-2 haplotypes (k, b, d and s). A minor determinant was found on the basis of a response to two peptides covering residues 12 to 29 and 18 to 37. They also demonstrated that B10. A (2R) mice (K^k 1-A^k 1-E^k D^k) recognize the major epitope between residues 48 and 54 following immunization with an MS2 fusion protein. A GST–E7 fusion protein used in conjunction with a Protein A–HPV-16 E7 fusion protein in CBA mice provided evidence for four distinct T cell epitopes spanning residues 20 to 29, 45 to 54, 60 to 79 and 85 to 94 (Comerford et al., 1991).

Our approach has been to purify fusion protein by affinity chromatography and to cleave this material to produce HPV-16 E7 itself (see Fig. 1). Immunization with the cleaved HPV-16 E7 protein failed to elicit a response to peptides in several experiments (data not shown). This may be due to a dose effect, because obtaining significant quantities of cleaved HPV E7 was difficult despite the relatively good yield of GST–E7 protein (10 mg/100 ml bacterial culture) and GST from affinity chromatography. Alternatively, GST may have promoted the immunogenicity of the HPV-16 E7 protein. It should be noted that mock-immunized mice mounted reduced but significant responses to GST and GST–E7 protein (see Fig. 2 legend), a finding consistent with the reported serological reactivity of normal mice to this antigen (Davern et al., 1987).

The absolute level of response to the peptides in our study was much lower than that reported by Tindle et al. (1991), which may reflect the difference in approach. The SI when using inclusion bodies as the immunogen was between 3 and 8 (Comerford et al., 1991), and thus more comparable to those reported here. The rather low levels of thymidine uptake with peptides, together with the variation in the peptide response profile of the various strains, makes it difficult to delineate precise T cell epitopes for each strain or haplotype. Nevertheless, the data point to five regions (residues 1 to 9, 17 to 32, 42 to 59, 62 to 77 and 87 to 98) on the HPV-16 E7 molecule which contain T cell epitopes. It is likely that these determinants are recognized by T helper cells in the context of major histocompatibility complex class II molecules, but this requires further investigation. There is good agreement between the regions defined here and the epitopes identified by Comerford et al. (1991). Only the first epitope (residues 1 to 9) represents a previously silent region because CBA mice do not appear to recognize these residues.

These experiments were performed with a view to using fusion proteins and peptides to identify T cell responses to HPV-16 E7 protein in man. Our mouse data indicate that there is not a single dominant epitope recognized when a fusion protein is used to induce a response to E7 protein. We have begun to assess the response of human peripheral blood lymphocytes to HPV-16 E7 protein and preliminary results suggest that, as in the mouse, a range of different epitopes are recognized.

Our data, like those of others, confirm that a wide variety of mouse strains recognize the HPV-16 E7 molecule. Allowing for the variation between experiments, it was evident that different strains recognized some but not necessarily all of the regions, possibly a result of immune response genes within or outside H-2. The lack of reactivity in BALB/c mice is puzzling because other H-2^d mice (B10. D2) respond to the HPV-16 E7 protein and Tindle et al. (1991) obtained a proliferative response to residues 44 to 62 after immunization of BALB/c mice with the peptide. However, Comerford (1991) did not observe significant responses to any E7 peptide in BALB/c mice after immunization with inclusion bodies containing the GST–E7 fusion protein. The implications of our results and those of Comerford et al. (1991) for the human immune response are twofold. First, many HLA haplotypes may be expected to respond to the HPV-16 E7 protein if, during the course of a natural infection, T lymphocytes encounter the antigen on an antigen-presenting cell. Second, some HLA haplotypes might be expected to respond only weakly or not at all. Furthermore, the data suggest that it might be efficacious to include the whole E7 molecule in a vaccine against HPV-16 rather than selected fragments or peptides.

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**References**


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