Abstract

We have reported previously that cottontail rabbit papillomavirus (CRPV) E8 gene immunization induced strong protection against virus challenge. In this study, we primed E8 gene vaccination with mouse granulocyte-macrophage colony-stimulating factor (mGM-CSF), a cytokine that induces differentiation and local recruitment of professional antigen-presenting cells. EIII/JC inbred rabbits were divided into four groups receiving vaccinations with the following constructs: mGM-CSF plus E8, mGM-CSF only, E8 only and vector only. After three immunizations at intervals of 3 weeks, rabbits were challenged with viral DNA at six scarified sites. Papillomas grew on all vaccinated rabbits 4 weeks after inoculation. At week 5, papillomas on four rabbits of mGM-CSF plus E8 and one of E8 only rabbits began to regress. At week 11, all the papillomas on rabbits in the GM-CSF plus E8 vaccination group regressed (regression rate = 100%); regression rates of the mGM-CSF only and E8 only vaccination groups were 50 and 25%, respectively. All papillomas on the vector immunized rabbits remained persistent until the end of the experiment (0%). Antibodies to mGM-CSF were detected in rabbit serum by Western blot. Rabbits vaccinated with E8 plus mGM-CSF or E8 only group had positive Delayed-type hypersensitivity (DTH) skin test to different E8 peptides. These results demonstrated that mGM-CSF could enhance the effects of E8 immunization in rabbits to CRPV infection through cell-mediated immune responses.

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1. Introduction

Papillomaviruses can induce warts in both cutaneous and mucosal tissues. More than 150 human papillomavirus (HPV) types have been identified genetically. A correlation between high-risk HPV infection and cervical cancer has been well documented, and HPV-associated cancers account for approximately 10% of cancers worldwide [1, 2]. Eradication of HPV-induced papillomas requires development of effective prophylactic and therapeutic vaccines.

The cottontail rabbit papillomavirus (CRPV)/rabbit model is a mature and powerful animal model for HPV vaccine development [3]. CRPV-induced papillomas progress to carcinoma in a pattern similar to those induced by high-risk HPVs. CRPV-induced papillomas may spontaneously regress, remain benign or become malignant. Both the viral and host genetic backgrounds play a role in papilloma evolution [4-6]. Thus, an additional strength of the CRPV/rabbit model for understanding host anti-viral immunity is the recent observation of variants that show different outcomes following infection [6, 7]. Such variants enable investigators to identify common and specific epitopes recognized by T-cells for construction of multi-epitope vaccines that represent effective strategies in other virus infection models [8].

DNA vaccination strategies with recent improvements have been used widely in preventive and therapeutic vaccination against both virus infections and experimental tumor growth [9-11]. Others and we have used DNA vaccination with various individual or recombinant CRPV genes for induction of host immunity against virus challenge [12-17].

The CRPV L1 gene alone can provide complete protection against virus challenge but only partial protection against viral DNA challenge [12] (Hu, unpublished observations). These observations indicate that the L1 gene provokes...
strong protective humoral responses and incomplete protective cell-mediated responses. For elimination of the papillomas, cell-mediated immune responses are crucial [18–22]. CRPV early genes (E1, E2, E6 and E7) stimulate protective T-cell mediated immune responses [14–16]. E6 and E7 genes are considered best targets for host immunity because of their up-regulated expression in malignant papillomas [23–25]. However, E6 gene vaccination provides only partial protection against virus challenge and E7 gene vaccination has no protective effect although it can induce strong T-cell mediated immune responses in vivo [15,16]. More recently, we found that E7 vaccination plays a role in a delay in malignant progression [14]. E1 and E2 gene vaccination provided good protection against both virus and viral DNA challenge [15]. Vaccination with a combination of E1, E2, E6 and E7 completely protected against virus challenge and also delayed the malignant onset of virus-induced papillomas [16,23,26]. These results indicated that multiple different epitopes on these early genes are required for eradicating benign and malignant papillomas. We previously reported that CRPVE8, a small gene which encodes a 50 amino acid protein and which is collagen with the E6 gene, was also an effective antigen against virus challenge in both outbred and inbred rabbits [27].

Immune responses generated by DNA vaccination are initiated by professional antigen-presenting cells (APCs) in vivo [11,28]. At the site of vaccination, cytokines play a critical regulatory role in the development of these immune responses by inducing maturation, activation, and local recruitment of langerhans cells and other APCs both in vivo and in vitro. In particular, granulocyte-macrophage colony-stimulating factor (GM-CSF) is an effective genetic adjuvant for DNA vaccination because GM-CSF increases T- and B-cell responses [29–33]. CRPV E6 gene immunization together with mouse GM-CSF priming leads to elimination of over 90% of virus-induced papillomas [18].

We have shown previously that E8 induced strong but incomplete protection against CRPV challenge [27]. Our unpublished observations). In this study, we evaluated a combination of mouse GM-CSF and E8 vaccination against viral DNA challenge in rabbits. Our results demonstrated that E8 vaccination provided partial protection against viral DNA challenge in inbred rabbits. mGM-CSF vaccines dramatically enhanced this response, leading to the complete eradication of papillomas around 8 weeks after viral DNA challenge. mGM-CSF vaccination alone also had a strong effect resulting in elimination of papillomas on half of the rabbits and suppressing papilloma outgrowth. Immunity against the E8 protein was also detectable using in vivo delayed-type hypersensitivity (DTH) tests with four synthesized E8 peptides. mGM-CSF priming led to an earlier onset of DTH responses to E8 peptides in animals immunized with a combination of E8 and mGM-CSF. These data demonstrated that GM-CSF is an effective adjuvant for E8 gene vaccination leading to papilloma regression in these animals.

2. Materials and methods

2.1. Preparation of constructs and micro-particles for gene-gun vaccination

The CRPV E8 genes was amplified by PCR and cloned into the V1Jns expression vector (generous gift of M.A. Liu, Merck & Co., Westpoint, PA) at the BglII site [27,34]. This plasmid was identified as E8V1Jns [27]. Mouse GM-CSF gene was amplified by PCR and cloned into the pVAX1 expression vector (Invitrogen, CA) and identified as mGM-CSF pVAX1. All genes inserted into the recombinant plasmid DNA constructs (E8V1Jns, mGM-CSF pVAX1) were confirmed by DNA sequence analysis and purified by QIAGEN Maxiprep kit and cesium chloride density gradient ultracentrifugation. The plasmid DNAs were prepared at 1 μg/μl and precipitated onto 1.6 μm-diameter gold micro-particles at a ratio of 1 μg of DNA/0.5 μg of gold particles as described by the manufacturer (Bio-Rad, Hercules, CA) and in previous studies [27].

2.2. Rabbit vaccination and DNA challenge

EIII/JC inbred rabbits were maintained in the animal facility of the Pennsylvania State University College of Medicine. All animal care and handling procedures were approved by the Institutional Animal Care and Use committee of the Pennsylvania State University. EIII/JC inbred rabbits were divided into four groups and immunized with E8V1Jns plus mGM-CSF pVAX1, E8V1Jns only, mGM-CSF pVAX1 only and V1Jns plus pVAX1, respectively. Inner ear skin sites of anesthetized rabbits were shaved and swabbed with 70% ethanol, then were bombarded with DNA/gold at 400 lb/in.² using a gene gun. A previous study had demonstrated that the peak influx of dendritic cells occurred 1–3 days after mGM-CSF inoculation and then gradually decreased [18]. We thus primed rabbits with mGM-CSF 3 days before E8 immunization at the first and second vaccination time points but co-inoculated with mGM-CSF and E8 for the final booster immunization. Animals were immunized for a total of three times at 3-week intervals. Each plasmid DNA was applied at a total dose of 20 μg per rabbit for each immunization. One week after the final booster immunization, rabbit back skin was pre-treated with a mixture of turpentine and acetone (T&A, 50:50, v/v) four times, every other day to make the skin hyperplastic [35,36]. Rabbits were challenged with wt CRPV DNA at six scarified back sites with 10 μg viral plasmid DNA per site. Papilloma measurements began 4 weeks after viral DNA challenge and continued weekly for 12 weeks.

2.3. Western blot

Blood was collected before and after DNA vaccination and serum prepared. In vitro translated mGM-CSF was separated by sodium dodecyl sulfate-polyacrylamide gel
electrophoresis (15%) and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked in 5% dry milk in PBS, and then incubated with rabbit sera at 1:100 dilutions. Peroxidase-conjugated swine anti-rabbit immunoglobulins (DAKO, Denmark) were used as second antibody. Membranes were developed with ECL + Plus western blotting detection system (Amersham, Pharmacia Biotech, UK). After about 2 min exposure, the films were developed using a Konica Medical film processor SRX-101 (Japan).

2.4. Peptide synthesis

Four peptides spanning the CRPV E8 gene (peptides 1–4) and one peptide from the rabbit oral papillomavirus (ROPV) E5 gene were synthesized and purified by Genemed Company (South San Francis, CA). Peptide 1 (MGPAETALYCY, aa 1–10) and peptide 2 (RLYKLAGSCVWQ, aa 35–50) were dissolved in conjugation solution (0.83 M sodium phosphate, 0.9 M NaCl, 0.1 M EDTA, pH 7.2) to make stock solutions (20 mg/ml) then diluted in sterile PBS prior to injection. Negative controls for peptides 1 and 2 was a peptide (SMGVLECTLGVWEC) from the ROPV E5 gene [37]; peptide 3 (YCLVLWIFIVTLLLL, aa 9–23) was dissolved in DMSO to make a stock solution (3.6 mg/ml), and then diluted in sterile PBS prior to injection. Controls for peptide 3 consisted of DMSO dissolved in PBS. Peptide 4 (LWLLWLRWLLR, aa 24–36) was dissolved in PBS. Control for peptide 4 was PBS.

2.5. Skin test

The procedure consisted of the intracutaneous injection of 0.03 ml (10 μg peptide) test solution into the outer ear skin as reported previously [27,38]. One week after the final booster immunization, peptide 1, peptide 2 and their control peptide were injected on different sites of one ear; peptides 3 and 4 and their control solution were injected into different sites of the second ear. Elizabethan collars were fitted to all rabbits after ear injections to prevent non-specific inflammation caused by scratching [38]. Ear swelling was monitored over 5 days and measured to the nearest 0.01 mm with a constant tension thickness gauge. All ear swelling data are represented as the differences between ear swelling for the test peptides and the corresponding control peptides. The ear swelling induration >0.1 mm was considered positive for DTH reaction. In addition, the intensity of erythema was documented.

2.6. Papilloma size determination and statistical analysis

Papilloma size was determined by calculating the cubic root of the product of length X width X height of individual papillomas in millimeters to obtain a geometric mean diameter (GMD). Data were represented as the means (±S.E.M.s) of the GMDs for each test group. Statistical significance of papilloma size was determined by unpaired t-test comparisons.

Regression frequencies occurring from different vaccinated groups were compared with each other using Fisher’s exact probability test for small samples.

3. Results

3.1. DNA-induced papillomas regressed in E8 vaccinated rabbits

Sixteen III/JC inbred rabbits were divided into four groups and vaccinated with E8 plus mGM-CSF, E8 alone, mGM-CSF alone and vector alone (Table 1). One rabbit in the vector alone group died before the papilloma measurement began. Therefore, the papilloma appearance sites for the vector only group were 18. All vaccinated rabbits grew papillomas at week 4. No protection was found in these vaccinated rabbits in different groups. No significant difference was detected for papilloma sizes between the groups at this time point. However, papillomas in the E8 plus mGM-CSF group of rabbits began to regress at week 5. All the papillomas in this group uniformly regressed around week 8. Papillomas on one of four rabbits vaccinated with E8 alone regressed; Papillomas on two rabbits vaccinated with mGM-CSF alone regressed around week 10. There were no papilloma regressions in rabbits receiving vector alone. Regression rates for rabbits vaccinated with E8 plus GM-CSF, E8 only, GM-CSF only and vector was 100, 25, 50 and 0%, respectively (Table 1). No significant difference was found when regression rates were compared between the E8 plus mGM-CSF and mGM-CSF only groups. However, the regression rate of the papillomas in the E8 vaccination group was significantly lower than that of the E8 plus mGM-CSF vaccination group ($P < 0.01$, Fisher exact test). The mean papilloma size of all three vaccination groups was significantly suppressed when compared with the vector control group (Fig. 1, $P < 0.01$, Fisher exact test). The mean papilloma size of all three vaccination groups was significantly suppressed when compared with the vector control group (Fig. 1, $P < 0.01$, Fisher exact test). The mean papilloma size of all three vaccination groups was significantly suppressed when compared with the vector control group (Fig. 1, $P < 0.01$, Fisher exact test).

Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Vaccine</th>
<th>Papilloma appearance</th>
<th>Regression rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Vector only</td>
<td>18/18</td>
<td>0%</td>
</tr>
<tr>
<td>2</td>
<td>E8 only</td>
<td>24/24</td>
<td>62/4</td>
</tr>
<tr>
<td>3</td>
<td>mGM-CSF only</td>
<td>24/24</td>
<td>50/4</td>
</tr>
<tr>
<td>4</td>
<td>E8 + mGM-CSF</td>
<td>24/24</td>
<td>100/0</td>
</tr>
</tbody>
</table>

*Regression papilloma sites/total papilloma sites.

1 One rabbit died after DNA challenge due to un-related causes.

2 $P = 0.01$, vs. vector group, Fisher’s exact test.

3 $P = 0.01$, vs. E8 + mGM-CSF group, Fisher exact test.

4 $P < 0.01$, vs. vector group, Fisher exact test.

5 $P = 0.05$, vs. E8 and E8 + mGM-CSF group, Fisher exact test.
Fig. 1. Papilloma growth curves following viral DNA infection. Means and standard errors of the geometric mean diameter (GMD) of all the papillomas on all rabbits of each group were plotted against time after viral DNA challenge. The papilloma size of rabbits vaccinated with E8 only and mGM-CSF was significantly suppressed compared with papillomas on rabbits vaccinated with vector ($P<0.05$, t-test). All papillomas on the group immunized with E8 plus mGM-CSF regressed ($P<0.001$, t-test).

No significant difference was found between mGM-CSF only and E8 only vaccinated rabbits (Fig. 1).

3.2. Humoral immune response initiated by mGM-CSF vaccination

Rabbit serum was collected 1 week after the final booster immunization and tested for anti-GM-CSF antibodies via western blot analysis (Section 2). Sera from individual rabbits were used as primary antibody (1:100). After incubation with secondary antibody, the membrane was developed. Positive bands were detected for sera from seven of eight rabbits immunized with mGM-CSF (data not shown). Rabbit sera from the vector only group were negative.

3.3. T-cell mediated immune responses

Specific cell-mediated immune responses to CRPV E8 were tested by DTH reactions using four peptides spanning the whole E8 protein (Table 2). In the E8 plus mGM-CSF vaccination group, one rabbit showed positive skin test reactions to all four peptides while the other three rabbits showed positive skin test reactions to at least one peptide at 48 h. In the E8 alone vaccination group, two rabbits showed positive reactions to peptides 3 while one rabbit showed a positive DTH response to peptide 2. One rabbit in the mGM-CSF vaccinated group had a positive DTH response to peptide 3 (data not shown). In contrast, none of the rabbits in the control group showed reactivity to any of the E8 and control peptides. Time-course analysis of DTH reactions to peptide 1 of the vaccinated rabbits indicated that peak reactions were at 48 h and began to decline around 120 h. A single E8 vaccinated rabbit however showed persistent DTH responses for the duration of measurements (Fig. 2). These observations showed that strong skin test reactions correlated with effective eradication of papillomas.

Fig. 2. DTH test results of peptide 1 on different groups of rabbits at different time points after peptide injection. Rabbits were tested 1 week after the final booster immunization. Rabbits in E8 plus mGM-CSF and E8 alone groups showed strong positive reactions to peptide 1 at 48 h after injection (A) while two rabbits of E8 group (B) remained positive at 96 h. None of the rabbits from the mGM-CSF (C) and vector (D) vaccination groups showed strong positive reaction at these time points.
4. Discussion

We previously found that the CRPV E8 protein was an effective antigen for induction of specific immunity against CRPV challenge in both NZW outbred and EIII/JC inbred rabbits. These earlier observations were confirmed and we additionally found that mGM-CSF dramatically enhanced the effect of E8 vaccination resulting in complete elimination of the CRPV DNA-induced papillomas.

Papillomavirus L1 and L2 vaccinations can provide complete protective immunity against virus infection when introduced as expression vectors [12,40]. These observations indicate that neutralizing antibodies induced by L1 and L2 immunization can completely block virions from entering host epithelial cells as no viral DNA can be detected in these vaccinated hosts [12,39,40]. However, cell-mediated rather than humoral immune responses are critical for eliminating the virus or viral DNA infected cells [20,41,42]. That’s to say, virus or viral DNA infected cells can be eradicated before they become clinically visible. Early genes such as E1, E2, E6, E7 and E8 vaccination stimulate T cell-mediated immune responses that are potentially important for these protective immuno-therapeutic responses [15,16,27].

Partial immunity following E8 vaccination (25% site regression) was detected in this experiment. In our previous studies, we established that the CRPV E8 gene is co-linear with the E6 gene and encodes a 50 amino acid hydrophobic protein with features similar to the E5 genes of both bovine papillomavirus type 1 (BPV-1) and most HPVs [43,44,53]. However, in the context of the CRPV genome, E8 has impact on papilloma outgrowth and host immune responses when used to vaccinate rabbits. Delayed-type hypersensitivity skin tests demonstrated that specific T-cell mediated immune responses had been elicited in vaccinated rabbits [27]. In the current study, we confirmed our previous observations that E8 vaccination alone induced T-cell mediated immunity against CRPV viral DNA infection. The small size and strong hydrophobic properties of E8 led to very low levels of expression and difficulties in protein detection in cell lines [27,34]. We hypothesized that E8 immune responses could be augmented by either enhancing levels of E8 expression and/or recruiting dendritic cells to the sites of E8 expression in vivo.

GM-CSF has been successfully applied as an adjuvant in many vaccination studies in different viral systems [18,29,31]. GM-CSF is an effective cytokine that can stimulate and recruit dendritic cells to the sites of antigen presentation in vivo. We found that E8 gene immunization was substantially augmented when co-inoculated with mouse GM-CSF. Although there was no protection against the viral DNA challenge, all four rabbits vaccinated with the combination of mGM-CSF and E8 were free of papillomas around the ninth week post viral DNA challenge. These data implied that mGM-CSF was effective at recruiting E8 antigen presenting cells which may have led to an increase in immune effector cells able to kill CRPV-infected papilloma cells, thus leading to subsequent regression.

GM-CSF has been shown to induce potent systemic anti-tumor immunity in hosts [45–50]. Previous studies have demonstrated that dendritic cells can take up DNA-coated beads and migrate to lymph nodes within 24 h [28,51,52]. Because GM-CSF itself can activate APCs and subsequently recruit dendritic cells, a strong adjuvant effect without any antigen is possible. In our current studies, two rabbits in the mGM-CSF only vaccination group developed strong immunity leading to regression of papillomas. We demonstrated expression of mGM-CSF in transiently transfected cell cultures before conducting DNA vaccinations (data not shown). To further identify expression in vivo, we tested for the presence of the antibody because mGM-CSF had been shown to be expressed successfully in vaccinated rabbit skin in a previously published study. However, in this previous study, antibody generation was not tested [18]. The investigators found a weak anti-papilloma response following
mGM-CSF vaccination alone with no significant difference between mGM-CSF and vector vaccination groups [18]. The contrasting results between this and our previous study may have resulted from the use of different expression vectors for mGM-CSF and viral genes in the two sets of experiments. In addition, our study challenged rabbits with viral DNA rather than infectious virions. Others and we have observed that protective immunity following vaccinations is in general strongest against viral DNA challenge versus CRPV virion challenge with the possible exception of E8 [15,27]. Taken together, our study showed that mGM-CSF vaccination had a strong impact on papilloma elimination and outgrowth.

In conclusion, E8 vaccination suppressed viral DNA induced papilloma growth in EIII/JC inbred rabbits and eliminated all papillomas when genetically primed with mGM-CSF expression vectors. These results are not only consistent with our previous findings but also provide increased evidence for the potential effect of cytokines as an adjuvant in vaccinations against papillomavirus infections.

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References


