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Short communication

Enhanced immune response to DNA-based HPV16L1 vaccination by costimulatory molecule B7-2

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Abstract

We have investigated whether co-injection of DNA encoding the costimulatory molecule B7-2 augments immune response to the major capsid protein L1 of the high-risk human papillomavirus type 16 (HPV16L1). While immunoglobulin G (IgG) specific to HPV16L1 was detected in sera from mice injected intramuscularly with pcDNA-L1 that encodes HPV16L1, a significantly increased level of IgG was found in sera from mice immunised with pcDNA-L1 in conjunction with pLXHDmB7-2 DNA. Levels of IgG in the anti-sera were correlated with the inhibitory activity of the murine erythrocyte hemagglutination caused by the virus-like particles (VLP) and the binding of VLP to HeLa cells. Moreover, splenic cells isolated from mice co-injected with pLXHDmB7-2 had stronger proliferation and more IFN-γ producing T cells ($CD4^+$ and $CD8^+$) when stimulated with HPV16 VLP compared with cells from mice that had received pcDNA-L1 alone and mice of the control groups. Furthermore, in footpad swelling test, mice co-immunised with pLXHDmB7-2 had greater skin thickness over those immunised with pcDNA-L1 alone or control mice. We conclude that co-injection of DNA encoding B7-2 can enhance both humoral and cellular immune responses elicited by DNA-based vaccination against HPV16 infection in mice.

Keywords: Human papillomavirus; DNA-vaccination; Costimulatory molecule

A growing body of evidence indicates that HPV, particularly type 16, is an important initiating agent of cervical carcinogenesis, causing up to a quarter of all cancer cases in women from developing countries (Lowy and Schiller, 1998a; Walboomers et al., 1999). Therefore, prevention of HPV16 infection is believed to be a potentially effective approach to control HPV-associated cervical carcinoma (Lowy and Schiller, 1998b; Coursaget and Munoz, 1999).

In recent years, DNA-based immunisation has emerged as a promising strategy to induce both humoral and cellular immunity against a variety of antigens (Bagarazzi et al., 1998; Benton and Kennedy, 1998). The basis for DNA vaccination rests on the observation that intramuscular inoculation of "plasmid or naked" DNA could induce protein expression within the muscle cells (Wolff et al., 1990). A further study showed that DNA vaccination could protect mice from influenza infection because of the induction of both antibody and $CD8^+$ cytotoxic T-lymphocytes (CTL) responses (Ulmer et al., 1993). DNA vaccination provides several advantages over current vaccines (Gurunathan et al., 2000). DNA vaccines mimic the effects of live attenuated vaccines in their ability to induce major histocompatibility complex (MHC) class I restricted $CD8^+$ T-cell responses, overcoming the safety concerns about the live vaccines and inability of eliciting cellular immunity by conventional protein-based vaccines. DNA vaccines can be easily manufactured to high quality with a cost-effective manner and stored with relative ease, suitable for economic transportation, which is especially beneficial in prevention of infectious diseases in developing countries. For these reasons, considerable efforts have been made to develop DNA-based vaccines to prevent HPV infection. Yet, trials on human so far have proven to be unsatisfactorily protective efficacy, presumably due to the low levels of antibodies or cellular responses (Tang et al., 1992; MacGregor et al., 1998).

T lymphocytes play a pivotal role in establishing acquired humoral and cellular immunity. To maintain the fidelity of the immune response, T lymphocytes must receive two distinct signals for their full activation (Salomon and Bluestone, 2001). An antigen-specific signal (first signal) is generated

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when the T cell receptor (TCR)-CD3 is engaged with antigen peptide–MHC complex on the surface of antigen-presenting cells (APC). A non-cognate (second signal) is provided by costimulatory factors (either soluble or on the surface of APC) when they interact with their receptors on T cells. The interaction between CD28 on T cell surface and the costimulatory molecule B7 family has now been proven of being the most prominent one among all costimulatory pathways identified (Salomon and Bluestone, 2001). In the B7 family, B7-2 (CD86), constitutively expressed on dendritic cells (DC) and rapidly upregulated on activated T and B cells, provides the dominant costimulatory signal, whereas B7-1 (CD80), which is only weakly expressed on DC, plays a more significant role in sustaining T cell activation (Schweitzer and Sharpe, 1998). With regards to DNA vaccination, several studies show that B7-2 is more potent than B7-1 to enhance cytotoxic T lymphocyte (CTL) responses (Kim et al., 1997; Iwasaki et al., 1997; Tsuji et al., 1997; Agadjanyan et al., 1999). In another study, B7-2 was found to be critical for generating both humoral and cellular immune responses against HIV-1 gp120 protein (Santra et al., 2000b). In light of these reports, we have specifically investigated the effect of B7-2 on DNA-based vaccination against HPV16.

Plasmid DNA of pcDNA-L1 that encodes HPV16L1 (Sun et al., 2000), pLXHDmB7-2, encodes the costimulatory molecule B7-2 (kindly provided by Dr. Lieping Chen at NIH), and pcDNA3.1 were purified and concentrated to 10 μg/μl for all preparations. pLXHD only differs from pcDNA3.1 at the promoter region so that only the latter was used as vector control. Twenty-four female C57BL/6 mice, 6–8 weeks of age (Institute of Chinese Medicine of Shaanxi Province), were randomly divided into four groups to receive intramuscular injections. pcDNA-L1 (100 μg/mouse) was administered to group 1, a mixture of pcDNA-L1 (100 μg/mouse) and B7-2 (50 μg/mouse, according to Santra et al., 2000a) to group 2, phosphate-buffered saline (PBS, 100 μl/mouse) to group 3 and pcDNA3.1 (100 μg/mouse) to group 4. To enhance DNA absorption, 50 μl of 25% bupivacaine was injected into the quadriceps femoris muscles at 72 h prior to the DNA injections. All mice were injected with the same materials three times at a 3-week interval. Prior to the first injection and 14 days after each injection, blood was taken from mice to detect HPV16L1 specific IgG by ELISA. After the final injection, footpad swelling assay was performed by intradermal injection of 10 μg of HPV16L1 VLP protein expressed from baculovirus-Sf9 insect cell system (Kirnbauer et al., 1993; kindly supplied by Dr. J.T. Schiller at NIH) and 10 μg of ovalbumin in the left and right footpads, respectively. Fourteen days thereafter, animals were retro-orbitally bled and sacrificed immediately. Spleens were removed to prepare cell suspensions to assess cell proliferation activity and to detect interferon (IFN)-γ producing T cells.

To detect IgG anti-HPV16, ELISA was carried out using 96-well plates coated with HPV16L1 VLP (0.5 μg per well) as described (de Gruijl et al., 1997). Sera to be tested were diluted 1:100 and applied to wells 100 μl each, and anti-mouse IgG conjugated with horseradish peroxidase (HRP) was used as secondary antibody. Tetramethylbenzidine was used for colour development, and the optical density (OD, 450 nm) was determined using a Universal Microplate Reader (BIO-TEC Instruments). Fig. 1 shows the collective ELISA data. Sera of groups 3 and 4 produced background reading around 0.2 in all time intervals. In contrast, sera from immunised mice increased the OD values over twofold (Student's *t*-test, $P = 0.00001$) after first injection. We therefore conclude that IgG specific to HPV16L1 were present in the sera of immunised mice. Noticeably, sera of group 1 showed a sharp increase in the levels of IgG after the first injection, but little or no increase after the second and third immunisations. This indicates that repeated injections with pcDNA-L1 alone did not further boost the level of anti-HPV16L1 antibody. In contrast, IgG levels measured in sera of group 2 were not only higher than those detected in sera of group 1 after the first immunisation ($P = 0.0069$), but also increased significantly after second and third immunisation ($P = 0.0044$ and $P = 0.0001$, respectively). This indicates that multiple co-injection of pLXHDmB7-2 can effectively boost the production of specific IgG elicited by pcDNA-L1.

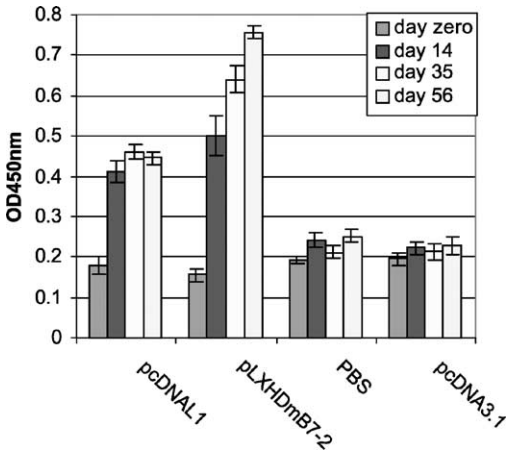

Fig. 1. Detection of serum IgG specific to HPV16L1 in mice pre- and post-immunisation. ELISA was carried out twice as outlined in the text. Each column represents the mean value of OD, 450 nm out of sera from six mice of each group at the time interval indicated.

HPV VLP causes hemagglutination of murine erythrocytes, which can be inhibited by neutralising antibodies against VLP (Roden et al., 1995). Hence, we tested whether the IgG from immunised mice could inhibit VLP-induced hemagglutination of erythrocytes (HAI). The procedure was as follows: (i) murine erythrocytes were isolated from blood by centrifugation (1000 × *g* for 5 min at 4 °C) and suspended in PBS to 1% (v/v) of its original volume; (ii) sera of each group were pooled and absorbed by 4 vol. of 25% (v/v) urine erythrocytes to eliminate non-specific agglutination, and then heated at 56 °C for 30 min to inactivate complements;

Table 1
Hemagglutination inhibition assay

Experimental group[a]	Inhibition activity at the dilution of[b]				
	1:200	1:400	1:800	1:1600	1:3200
pcDNA-L1	+++	++	±	−	−
pcDNA-L1 and pLXHDmB7-2	+++	+++	++	±	−
PBS	−	−	−	−	−
pcDNA3.1	−	−	−	−	−

[a] Sera from each group were pooled for the assay.
[b] Maximal inhibition (non-hemagglutination) is indicated as '+++' and maximal hemagglutination (non-inhibition) is expressed as '−'.

(iii) 50 μl of serial diluted serum was mixed with 50 μl of VLP stock solution (600 μg/ml in PBS–1%BSA) and applied per well in a 96-well plate. After a 1-h incubation at room temperature, 50 μl of 1% erythrocyte suspension was added to each well and plates were incubated at 4 °C for 3 h. Compared to the maximal HA produced by VLP stock solution (50 μl plus 50 μl PBS + 50 μl erythrocytes), sera from immunised mice showed HAI activity of which sera of group 2 were stronger (Table 1, Fig. 2). In contrast, sera of groups 3 and 4 had no HAI activity.

To further test the neutralising activity of the anti-sera, we explored our observation that HPV16L1 VLP can bind to HeLa cells (unpublished). The cell bound VLP can be captured by rabbit anti-HPV16L1 VLP IgG (a kind gift from Dr. J.T. Schiller). Following incubation with anti-rabbit IgG conjugated with HRP and the addition of substrate diaminobenzidine, cells bound with VLP turned to brown (Fig 3a). When VLP pre-incubated with neutralising antibody prior application to the cell monolayers, VLP no longer bound to the cells. By using this procedure, we demonstrated that pre-treatment with sera from immunised mice could inhibit VLP binding to HeLa cells. Pooled sera of group 2 can inhibit VLP binding at a dilution of 1:400 (Fig. 3c) while pooled sera of group 1 only showed inhibition activity at a dilution of 1:100 (data not shown). In contrast, VLP pre-treated with sera of group 3 (data not shown) and group 4 (Fig. 3d) did not prevent the binding of VLP to HeLa cells.

Next, we tested the proliferative activity of splenocytes isolated from different groups in response to VLP stimulation in vitro. Cell suspension was adjusted to 1×10^5 per 200 μl in RPMI1640 medium supplemented with 10% foetal calf serum (FCS, GibcoBRL) and seeded in 96-well U-bottom plate. HPV16L1VLP was added to a final concentration of 1 μg/ml. After incubation for 7 days at 37 °C under 5% CO_2, cells were pulsed with 0.5 μCi-^{3}H-TdR (Atomic Energy Physics Institute of Chinese Academy). Eighteen hours later, cells were harvested onto glass-fibre to measure the incorporated radioactivity using a β-scintillation counter (262-Firm, Xi'an, China). As shown in Fig. 4, splenocytes from all immunised mice proliferated in response to HPV16VLP. In contrast, splenocytes of groups 3 and 4 did not respond. Noticeably, however, splenocytes of group 2 had a higher proliferative activity compared with

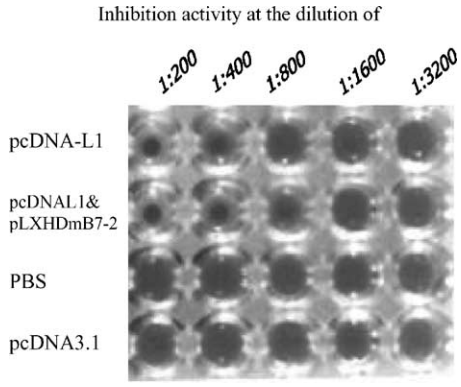

Fig. 2. Hemagglutination inhibition assay. A total of 50 μl of serial diluted serum and 50 μl of VLP (600 μg/ml) were incubated for 1 h at room temperature. Then, 1% mouse erythrocytes (50 μl per well) were added and incubated for another 3 h at 4 °C. The negative control, where PBS was used as a substitute for immunised serum, is indicated.

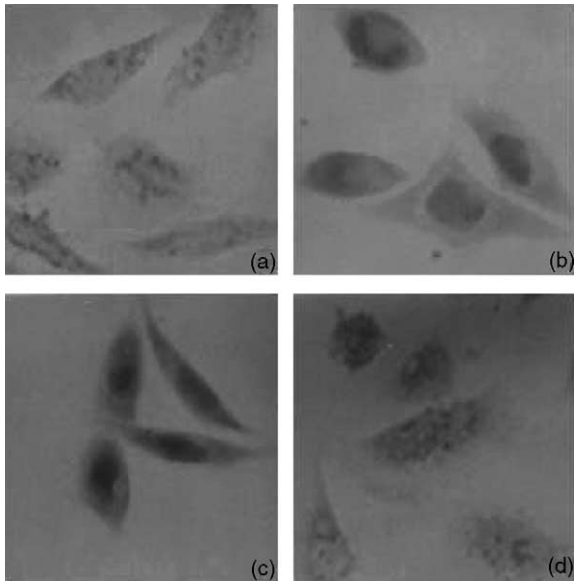

Fig. 3. HeLa cell binding assay. (a) HeLa cells infected with HPV16L1 VLP; (b) uninfected HeLa cells; (c) HeLa cells infected with HPV16L1 VLP that were pre-treated with serum from pooled sera of group 2 (1:400 dilution); (d) HeLa cells infected with HPV16L1 VLP that were pre-treated with pooled sera of group 4.

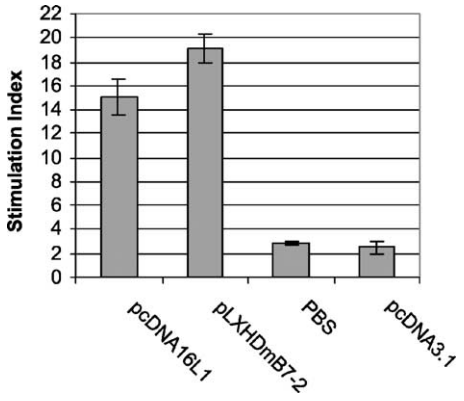

Fig. 4. Specific proliferation of splenocytes induced by HPV16 VLP. Stimulatory index represents the mean radioactive counts of cells from six mice of each group detected by a β-scintillation counter.

those of group 1 ($P = 0.002$), indicating that co-injection of pLXHDmB7-2 augments priming to HPV16VLP.

We further analysed populations of IFN-γ producing $CD4^+$ and $CD8^+$ T cells in splenocytes isolated from all groups by fluorescence activated cell sorting (FACS). Splenocytes were adjusted to $10^5\,ml^{-1}$ and treated with Monensin (Sigma) to block IFN-γ secretion. After incubation at 37 °C for 2 h, cells were stained with Phycoerythrin (PE)-labelled anti-CD4 or anti-CD8 monoclonal antibodies followed by Fluorescein isothiocyanate (FITC)-labelled anti-IFN-γ monoclonal antibody (Coulter Co.). Cells were then analysed with an EPICS®ELITE flow cytometer (Coulter Co.). As shown in Fig. 5, mice of group 1 had marginal higher numbers of IFN-γ secreting $CD4^+$ cells compared with mice of groups 3 and 4 ($P = 0.077$ and $P = 0.015$, respectively), but had significantly increased IFN-γ secreting $CD8^+$ cells ($P = 0.00002$). Strikingly,

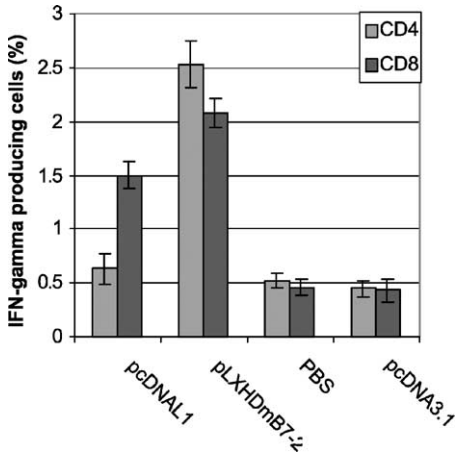

Fig. 5. Flow cytometry analysis of IFN-γ producing $CD4^+$ and $CD8^+$ splenocytes. $CD4^+$ and $CD8^+$ cells were labelled in red, of which IFN-γ producing cells were additionally labelled in green as outlined in the text. Approximately 10,000 $CD4^+$ and $CD8^+$ cells from each mouse were analysed and the mean percentage (%) of the IFN-γ producing cells is indicated.

Table 2
Footpad swelling response

Experimental group	Thickness (10^{-2} mm)	
	HPV16L1 VLP	Ovalbumin
pcDNA-L1	9.7 ± 1.16	2.9 ± 0.71
pcDNA-L1 and pLXHDmB7-2	16.6 ± 0.78	2.7 ± 0.86
PBS	2.8 ± 0.85	2.9 ± 0.60
pcDNA3.1	3.0 ± 0.63	3.1 ± 0.65

mice of group 2 had significantly higher numbers of IFN-γ producing cells of both $CD4^+$ and $CD8^+$ compared with group 1 ($P = 0.000002$ and $P = 0.00066$, respectively). Thus, while both immunised groups may have established $CD8^+$ T cell mediated CTL response, group 2 mice likely had a stronger cellular immunity of a broad spectrum due to the presence of higher numbers of IFN-γ producing $CD4^+$ cells. At the same time, group 2 mice possessed high levels of specific IgG (Fig. 1), indicating that they had developed well balanced humoral and cellular immune responses.

Footpad swelling test, a classic delayed-type hypersensitivity (DTH) assay, was used in a further attempt to investigate cellular response following DNA immunisation. As shown in Table 2, injection of pcDNA-L1 alone was sufficient to cause footpad swelling compared with control groups that had received either PBS or pcDNA3.1 ($P < 0.05$), or an unrelated protein, ovalbumin. Again, co-injection of mice with pLXHDmB7-2 resulted in a more significant swelling ($P < 0.001$). Histological examination of sections of the paws revealed more intense infiltration of mononuclear cells in the dermis of group 2 compared with those of group 1, and there was hardly any cell infiltration in tissues of groups 3 and 4 (data not shown). These results are consistent with the current theory that DTH is modulated by both $CD4^+$ and $CD8^+$ cells through their secretion of IFN-γ, a potent stimulator of macrophages (Black, 1999). More importantly, memory T cells are believed to be absolutely required for DTH (Black, 1999), hence a lasting immunity may have been established through the DNA vaccination protocol described here.

Several studies have shown the enhancement of CTL responses by co-injection of B7-2 DNA (Kim et al., 1997; Iwasaki et al., 1997; Tsuji et al., 1997; Agadjanyan et al., 1999). By using the B7-2 deficient mice, a more recent study demonstrated the necessity of B7-2 in generating humoral and cellular immunity in the case of DNA vaccination against HIV gp120 antigen (Santra et al., 2000b). Our data agree in general with the latter study, which, however, showed that co-injection of B7-2 DNA in wild-type BALB/c mice hardly affects antibody production. The difference between our data and those of the latter study can be due to different levels of B7-2 expression in the animals used (BALB/c versus C57BL/6) and the immunogenicity of the antigens (HIV gp 120 versus HPV16L1). The conclusion is, therefore, the same: B7-2 is critical for establishing humoral and cellular immunity following DNA-based vaccination.

Although in vivo models to evaluate the protective efficacy of the vaccination regimes are lacking, the heightened specific neutralising IgG antibodies may effectively prevent the binding of VLP to host cells and increased $CD8^+$ and $CD4^+$ T cell activities may exert cytotoxicity to those host cells that are infected by the virus (Volpers et al., 1995). Our findings should be useful in the development of future prophylactic and therapeutic DNA-based strategies not only for HPV infections but also for infections caused by other micro-organisms.

Acknowledgements

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