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Study of Cell-mediated Response in Mice by HPV16 L1 Virus-like Particles Expressed in *Saccharomyces cerevisiae*

WOO, MI-KYUNG[1], SOOK-JIN HUR[2], SUENIE PARK[3], AND HONG-JIN KIM[1]*

[1]*College of Pharmacy, Chung Ang University, Seoul 156-756, Korea*
[2]*Korea Food and Drug Administration, Seoul 122-704, Korea*
[3]*National Institute of Toxicological Research, Seoul 122-704, Korea*

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Abstract The first vaccine against human papillomaviruses (HPV) formulated with HPV16 L1 virus-like particles (VLPs) produced in yeast was approved by the FDA in June 2006. Nevertheless, there have been few studies of the immunogenicity in mice of VLPs. In this study, we evaluated the cell-mediated immune response to VLPs produced in *Saccharomyces cerevisiae*. After immunization of mice with HPV16 L1 VLPs, we measured splenocytes proliferation and the levels of IFNγ, IL2, IL4, and IL5. Splenocytes proliferation was significantly increased and a mixed Th1/Th2 response was indicated. IgG subtype immunoresponses were strongly induced and IgG1 titers were higher than those of IgG2a.

Keywords: Human papillomavirus, virus-like particles, splenocytes proliferation, cytokines, Th1/Th2 response

Cervical cancer is the second most common cancer in women worldwide and there are 200,000 cervical cancer-related deaths each year [19, 20, 24]. HPV16, 18, 31, and 45 are major causative factors of cervical cancers, and HPV16 is the most frequent type [1]. A prophylactic vaccine against HPV has been derived from HPV virus-like particles (VLPs). HPVs encode a major capsid protein (~55 kDa), L1, that can self-assemble into VLPs in the absence of other viral gene products. These VLPs are structurally similar to native HPV virions [6, 9, 13, 21–23]. Recombinant HPV16 L1 VLPs have been generally made in yeast and insect cells [15, 21]. Yeast-cell-derived HPV16 L1 VLP vaccination has been shown to induce Th1- and Th2-mediated cytokines and cytotoxic T lymphocyte responses in chimpanzees and humans [3, 18], but there is no report of Th1- and Th2-type immune responses to yeast-derived HPV16 L1 VLPs in the mouse model. Therefore, it seems important to study cell-mediated immunity induced by yeast-expressed HPV16 L1 VLPs in the mouse model. In the present study, we measured splenocytes proliferation, levels of cytokines IFNγ, IL2 (Th1-response), IL4 and IL5 (Th2-response), and IgG1 and IgG2a titers in response to immunization with HPV16 L1 VLPs.

Recombinant HPV16 L1 VLPs were prepared in a yeast system as described previously [5, 10, 12, 14, 22]. Ten mice were immunized by subcutaneous injection with 5 μg of HPV16 L1 VLPs with the Freund's complete adjuvant. After 3 weeks, all the mice were boosted twice with the same amount of VLPs adsorbed to Freund's incomplete adjuvant (Sigma, U.S.A.). Five control mice received PBS under the same conditions. To measure the splenocytes proliferation and cytokines response to VLPs, mice were sacrificed ten days after the last immunization and their spleens removed [11]. The splenocytes were resuspended in RPMI-1640 medium (20 mM HEPES, 10% fetal calf serum, 24 mM sodium bicarbonate, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin), and seeded at 2×10^5 cells per well in flat-bottomed 96-well plates. The splenocytes were incubated with HPV16 L1 VLPs (1.5 μg/well) for 6 days at 37°C.

To measure the proliferation response to VLPs, the MTT assay was used [8, 17]. The cells were pulsed with thiazolyl blue tetrazolium bromide (200 μg/well, Sigma, U.S.A.) for the final 4 h of incubation, supernatants were removed, DMSO (Sigma, U.S.A.) was added to each well, and absorbance was read at 540 nm. As shown in Fig. 1, the proliferation of splenocytes in immunized mice was significantly increased compared with the nonimmunized mice. After 6 days of incubation, the splenocytes from the mice immunized with VLPs had increased to 10×10^5 cells per well (5 fold), whereas the control splenocytes from mice injected with PBS had only increased to 4×10^5 cells per well (Fig. 1).

To measure cytokine production response to VLPs, each cytokine was measured in culture supernatants on days 4, 5, and 6. Levels of IFNγ, IL2, IL4, and IL5 were measured

*Corresponding author
Phone: 82-2-820-5613; Fax: 82-2-816-7338;
E-mail: hongjink@cau.ac.kr

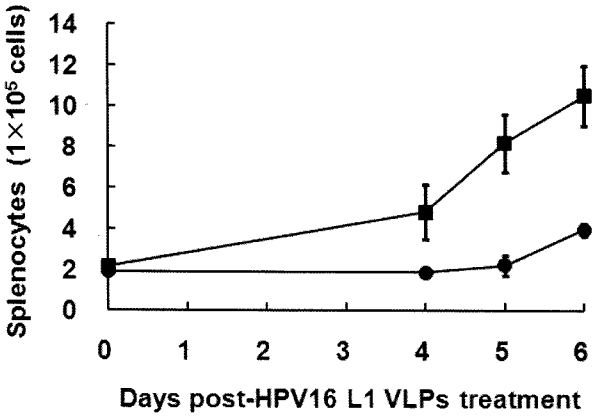

Fig. 1. Splenocytes proliferation in mice immunized with HPV16 L1 VLPs.
The immunized (■) and nonimmunized (●) groups were subcutaneously injected three times with HPV16 L1 VLPs (5 μg) and PBS, respectively. Splenocytes were isolated from the mice and incubated for 6 days with HPV16 L1 VLPs (1.5 μg/well). Proliferation of the splenocytes was measured with the MTT assay. Values are averages ±SD of results for the immunized group (n=10) and nonimmunized group (n=5).

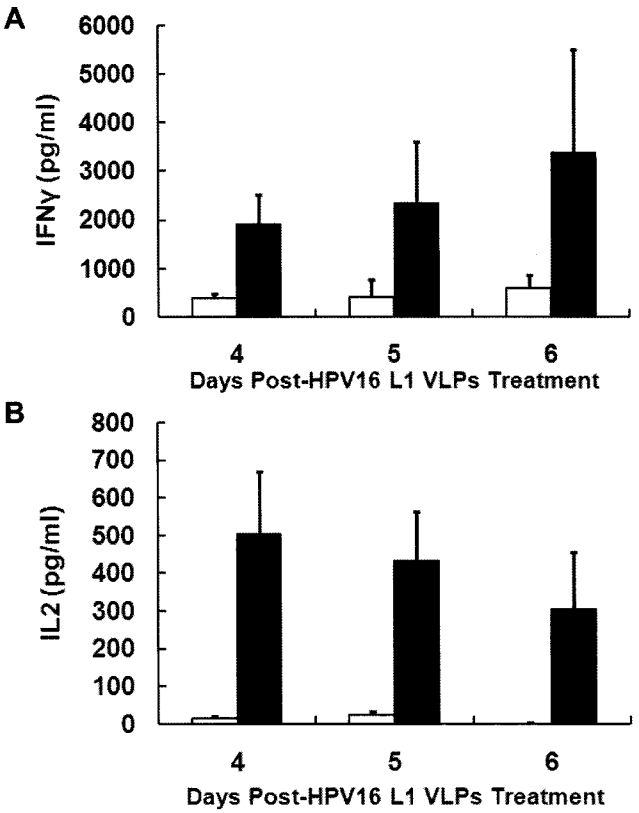

Fig. 2. Th1-type cytokine responses to HPV16 L1 VLPs.
Splenocytes from the mice injected with HPV16 L1 VLPs (solid bars) or PBS (open bars) were incubated for 6 days with HPV16 L1 VLPs (1.5 μg/well). IFNγ (**A**) and IL2 (**B**) in the culture supernatants were measured by sandwich ELISA on days 4, 5, and 6. Bars are averages ±SD of IFNγ and IL2 from the immunized group (n=10) and nonimmunized group (n=5). P<0.05.

by sandwich ELISA, according to the manufacturer's instructions (BD OptIEA Set, BD Biosciences, U.S.A.). As shown in Fig. 2, the immunized splenocytes produced significantly higher levels of Th1-type cytokines (IFNγ, IL2) than the nonimmunized splenocytes (Fig. 2). In the immunized group, IFNγ was detected at 1,900 pg/ml to 3,380 pg/ml, and IL2 was detected at 300 pg/ml to 400 pg/ml. In control mice, low levels of IFNγ (590 pg/ml) and IL2 (<20 pg/ml) were detected. In addition, the immunized group produced high levels of Th2-type cytokines (IL4, IL5); IL4 and IL5 were detected up to 210 pg/ml and 1,180 pg/ml, respectively (Fig. 3). These results show that the mice immunized with HPV16 L1 VLPs produced Th1 and Th2 responses.

To confirm the induction of a mixed Th1 and Th2 response to HPV16 L1 VLPs, we measured IgG1 (Th2) and IgG2a (Th1) in the sera of immunized mice by ELISA. The ELISA plates were coated with 100 ng of VLPs and blocked with 2% BSA for 1 h at room temperature. The wells were incubated with serial dilutions of mouse sera for 1 h at 37°C. The secondary antibodies, goat anti-mouse IgG1 (Zymed, U.S.A.) and IgG2a (Serotec, U.K.), which were diluted 1:2,000, were added to the wells and the plates were incubated at 37°C for 1 h. The unbound secondary antibody was removed by washing, and bound antibody was stained with substrate and absorbance read at 492 nm. As shown in Table 1, we observed a high IgG1 titer (23,000 to 84,000) in all sera from immunized mice but no VLP-specific IgG1 (<100) in those from nonimmunized mice. High levels of IgG2a (12,000 to 43,000) were also detected in the sera from immunized mice. However, the levels of IgG2a (<100) were not detected in sera from the control group. These results suggest that HPV16 L1 VLPs expressed in yeast are able to induce the Th1/Th2 mixed immune response.

We found that the proliferation of splenocytes (Fig. 1), and supernatant IFNγ and IL2 levels (Fig. 2) were increased by immunization, indicating that cell-mediated immune responses were induced. The production of IFNγ and IL2 was presumably induced by the proliferation of CD8+ and CD4+ T cells. Moreover, costimulation of Th1- and Th2-type cytokines was confirmed by the increased levels of IFNγ, IL2, IL4, and IL5 (Fig. 2 and Fig. 3).

In the case of insect-cell-derived VLPs, Dupuy *et al.* [2] reported that three immunizations of mice with 5 μg of HPV16 L1 VLPs induced only Th1-type cytokines (IFNγ, IL2), whereas Marais *et al.* [16] reported that three immunizations with 10 μg of HPV16 L1 VLPs induced both Th1- and Th2-type cytokines. In the present study, although the mice received three immunizations with only 5 μg of HPV16 L1 VLPs, both types of immune response were observed.

IFNγ induces IgG2a production and inhibits IgG1 production, whereas IL4 has the opposite effect [4]. Therefore,

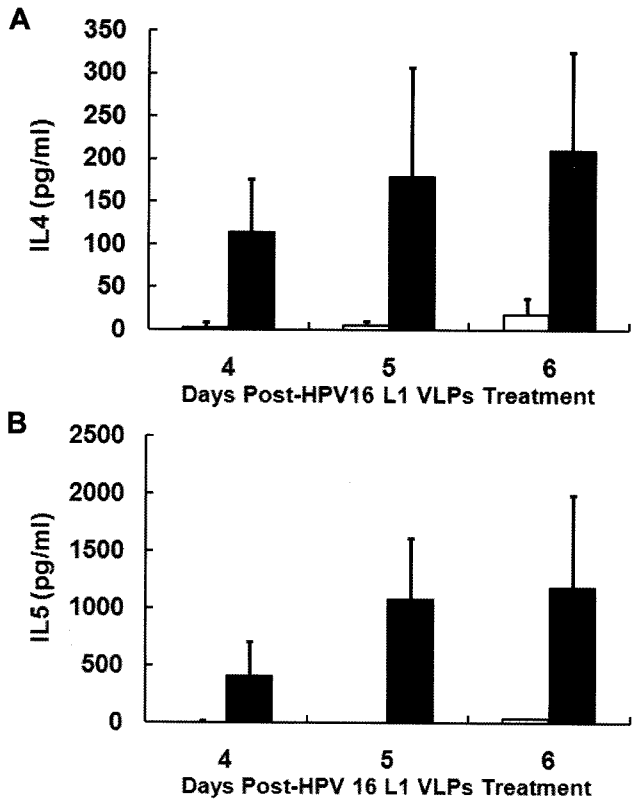

Fig. 3. Th2-type cytokine responses to HPV16 L1 VLPs. Splenocytes were incubated as in the legend to Fig. 2, and supernatants IL4 (**A**) and IL5 (**B**) were measured by sandwich ELISA on days 4, 5, and 6. Bars are averages ±SD of IL4 and IL5 from the immunized group (n=10) and nonimmunized group (n=5). P<0.05.

Table 1. IgG1 and IgG2a responses to HPV16 L1.

Group	Mouse ID No.	IgG subclasses[c]	
		IgG1	IgG2a
Immunized[a]	1	28,000	12,000
	2	48,000	19,000
	3	73,000	13,000
	4	62,000	17,000
	5	28,000	17,000
	6	84,000	16,000
	7	23,000	43,000
	8	31,000	24,000
	9	44,000	21,000
	10	35,000	16,000
Unimmunized[b]	11	<100	<100
	12	<100	<100
	13	<100	<100
	14	<100	<100
	15	<100	<100

[a]The immunized group (mouse ID Nos. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10) was subcutaneously injected with HPV16 L1 VLPs (5 μg).
[b]The nonimmunized group (mouse ID Nos. 11, 12, 13, 14, 15) was subcutaneously injected with PBS.
[c]IgG1 and IgG2a in the sera of the immunized and nonimmunized groups measured by sandwich ELISA.

the titer of IgG1 is higher than that of IgG2a when Th2-type cytokines preponderate, and lower when Th1-type cytokines preponderate [4]. In our study, the mean titers of IgG1 and IgG2a were 45,600 (±20,970) and 19,800 (±8,880), respectively, showing that there was a greater Th2-type than Th1-type immune response (Table 1).

We conclude that HPV16 L1 VLPs expressed in yeast induce a mixed Th1/Th2 immune response in mice and that the Th2-type response is stronger. The first HPV vaccine (Gardasil, Merck) formulated with HPV16 L1 VLPs produced in yeast was approved by the FDA in June 2006 [7]. We believe that our findings will contribute to the standardization and validation of the next generation of HPV vaccines in the mouse model.

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References

1. Bosch, F. X., M. M. Manos, N. Munoz, M. Sherman, A. M. Jansen, J. Peto, M. H. Schiffman, V. Moreno, R. Kurman, and K. V. Shah. 1995. Prevalence of human papillomavirus in cervical cancer: A worldwide perspective. International Biological Study on Cervical Cancer (IBSCC) Study Group. *J. Natl. Cancer Inst.* **87:** 796–802.
2. Dupuy, C., D. Buzoni-Gatel, A. Touze, P. Le Cann, D. Bout, and P. Coursaget. 1997. Cell-mediated immunity induced in mice by HPV 16 L1 virus-like particles. *Microb. Pathog.* **22:** 219–225.
3. Emeny, R. T., C. M. Wheeler, K. U. Jansen, W. C. Hunt, T. M. Fu, J. F. Smith, S. MacMullen, M. T. Esser, and X. Paliard. 2002. Priming of human papillomavirus type 11-specific humoral and cellular immune responses in college-aged women with a virus-like particle vaccine. *J. Virol.* **76:** 7832–7842.
4. Finkelman, F. D., J. Holmes, I. M. Katona, J. F. Urban, M. P. Beckmann, L. S. Park, K. A. Schooley, R. L. Coffman, T. R. Mosmann, and W. E. Paul. 1990. Lymphokine control of *in vivo* immunoglobulin isotype selection. *Annu. Rev. Immunol.* **8:** 303–333.
5. Ha, C. H., K. H. Lim, S. H. Jang, C. W. Yun, H. D. Paik, S. W. Kim, C. W. Kang, and H. I. Chang. 2006. Immune-enhancing alkali-soluble glucans produced by wild-type and mutant *Saccharomyces cerevisiae*. *J. Microbiol. Biotechnol.* **16:** 576–583.
6. Hagensee, M. E., N. Yaegashi, and D. A. Galloway. 1993. Self-assembly of human papillomavirus type 1 capsids by expression of the L1 protein alone or by coexpression of the L1 and L2 capsid proteins. *J. Virol.* **67:** 315–322.
7. Inglis, S., A. Shaw, and S. Koenig. 2006. Chapter 11: HPV vaccines: Commercial research and development. *Vaccine* **24Suppl3:** S99–S105.

8. Khajuri, A., A. Gupta, F. Malik, S. Singh, J. Singh, B. D. Gupta, K. A. Suri, P. Suden, V. K. Srinivas, K. Ella, and G. N. Qazi. 2007. A new vaccine adjuvant (BOS 2000), a potent enhancer mixed Th1/Th2 immune responses in mice immunized with HBsAg. *Vaccine* **25:** 4586–4594.
9. Kim, E., H. Y. Poo, M. H. Sung, and C. J. Kim. 2005. Mature HIV-like particles produced from single Semliki Forest virus-derived expression vector. *J. Microbiol. Biotechnol.* **15:** 1229–1239.
10. Kim, H. C., H. J. Kim, W. B. Choi, and S. W. Nam. 2006. Inulooligosaccharide production from inulin by *Saccharomyces cerevisiae* strain displaying cell-surface endoinulinase. *J. Microbiol. Biotechnol.* **16:** 360–367.
11. Kim, H. Y., J. O. Yang, and G. E. Ji. 2005. Effect of bifidobacteria on production of allergy-related cytokines from mouse spleen cells. *J. Microbiol. Biotechnol.* **15:** 265–268.
12. Kim, S. N., H. S. Jeong, S. N. Park, and H. J. Kim. 2007. Purification and immunogenicity study of human papillomavirus type 16 L1 protein in *Saccharomyces cerevisiae*. *J. Virol. Methods* **139:** 24–30.
13. Kirnbauer, R., F. Booy, N. Cheng, D. R. Lowy, and J. T. Schiller. 1992. Papillomavirus L1 major capsid protein self-assembles into virus-like particles that are highly immunogenic. *Proc. Natl. Acad. Sci. USA* **89:** 12180–12184.
14. Lee, J. S., J. Yu, H. J. Shin, Y. S. Kim, J. K. Ahn, C. K. Lee, H. Y. Poo, and C. J. Kim. 2005. Expression of hepatitis C virus structural protein in *Saccharomyces cerevisiae*. *J. Microbiol. Biotechnol.* **15:** 767–771.
15. Lowe, R. S., D. R. Brown, J. T. Bryan, J. C. Cook, H. A. George, K. J. Hofmann, W. M. Hurni, J. G. Joyce, E. D. Lehman, H. Z. Markus, M. P. Neeper, L. D. Schultz, A. R. Shaw, and K. U. Jansen. 1997. Human papillomavirus type 11 (HPV-11) neutralizing antibodies in the serum and genital mucosal secretions of African green monkeys immunized with HPV-11 virus-like particles expressed in yeast. *J. Infect. Dis.* **176:** 1141–1145.
16. Marais, D., J. A. Passmore, J. Maclean, R. Rose, and A. L. Williamson. 1999. A recombinant human papillomavirus (HPV) type 16 L1-vaccinia virus murine challenge model demonstrates cell-mediated immunity against HPV virus-like particles. *J. Gen. Virol.* **80:** 2471–2475.
17. Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assay. *J. Immunol. Methods* **65:** 55–63.
18. Palker, T. J., J. M. Monteiro, M. M. Martin, C. Kakareka, J. F. Smith, J. C. Cook, J. G. Joyce, and K. U. Jansen. 2001. Antibody, cytokine and cytotoxic T lymphocyte responses in chimpanzees immunized with human papillomavirus virus-like particles. *Vaccine* **19:** 3733–3743.
19. Parkin, D. M., P. Pisani, and J. Ferlay. 1999. Estimates of worldwide incidence of 25 major cancers in 1990. *Int. J. Cancer* **80:** 827–841.
20. Pisani, P., D. M. Parkin, F. Bray, and J. Ferlay. 1999. Estimates of the worldwide mortality from 25 cancers in 1990. *Int. J. Cancer* **83:** 18–29.
21. Rose, R. C., W. Bonnez, R. C. Reichman, and R. L. Garcea. 1993. Expression of human papillomavirus type 11 L1 protein in insect cells: *In vivo* and *in vitro* assembly of virus-like particles. *J. Virol.* **67:** 1936–1944.
22. Sasagawa, T., P. Pushko, G. Steers, S. E. Gschmeissner, M. A. Hajibagheri, J. Finch, L. Crawford, and M. Tommasino. 1995. Synthesis and assembly of virus-like particles of human papillomaviruses type 6 and type 16 in fission yeast *Schizosaccharomyces pombe*. *Virology* **206:** 126–135.
23. Volpers, C., P. Schirmacher, R. E. Streeck, and M. Sapp. 1994. Assembly of the major and the minor capsid protein of human papillomavirus type 33 into virus-like particles and tubular structures in insect cells. *Virology* **200:** 504–512.
24. Walboomers, J. M., M. V. Jacobs, M. M. Manos, F. X. Bosch, J. A. Kummer, K. V. Shah, P. J. Snijders, J. Peto, C. J. Meijer, and N. Munoz. 1999. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *J. Pathol.* **189:** 12–19.