Influence of Immunity Induced at Priming Step on Mucosal Immunization of Heterologous Prime-Boost Regimens

Seong-Kug Eo

Department of Microbiology, College of Veterinary Medicine and Bio-Safety Institute of Chonbuk National University, Jeonju, Korea

ABSTRACT

Background: The usefulness of DNA vaccine at priming step of heterologous prime-boost vaccination led to DNA vaccine closer to practical reality. DNA vaccine priming followed by recombinant viral vector boosting via systemic route induces optimal systemic immunity but no mucosal immunity. Mucosal vaccination of the reversed protocol (recombinant viral vector priming-DNA vaccine boosting), however, can induce both maximal mucosal and systemic immunity. Here, we tried to address the reason why the mucosal protocol of prime-boost vaccination differs from that of systemic vaccination. **Methods:** To address the importance of primary immunity induced at priming step, mice were primed with different doses of DNA vaccine or coadministration of DNA vaccine plus mucosal adjuvant, and immunity including serum IgG and mucosal IgA was then determined following boosting with recombinant viral vector. Next, to assess influence of humoral pre-existing immunity on boosting $CD8^+$ T cell-mediated immunity, $CD8^+$ T cell-mediated immunity in B cell-deficient (μK/O) mice immunized with prime-boost regimens was evaluated by CTL assay and IFN-γ -producing cells. **Results:** Immunity primed with recombinant viral vector was effectively boosted with DNA vaccine even 60 days later. In particular, animals primed by increasing doses of DNA vaccine or incorporating an adjuvant at priming step and boosted by recombinant viral vector elicited comparable responses to recombinant viral vector primed-DNA vaccine boosted group. Humoral pre-existing immunity was also unlikely to interfere the boosting effect of $CD8^+$ T cell-mediated immunity by recombinant viral vector. **Conclusion:** This report provides the important point that optimally primed responses should be considered in mucosal immunization of heterologous prime-boost regimens for inducing the effective boosting at both mucosal and systemic sites. **(Immune Network 2003;3(2):110-117)**

Key Words: DNA vaccine, Heterologous prime-boost vaccination, Mucosal immunity

Introduction

The discovery that DNA vaccination can induce Ab and cell-mediated responses to a variety of bacterial, viral and parasitic Ags opened a new approach to the control of infectious diseases (1). The level of protection afforded by DNA vaccines, however, is often inferior to the efficacy of conventional subunit vaccine or attenuated live vaccine (1-4). This led to numerous attempts to augment and/or modulate the immune responses to DNA vaccine (5-7). In recent, the protective efficacy of DNA vaccine increased if DNA vaccines were used to prime, and either recombinant protein (8,9) or recombinant viral vector encoding Ag (10,11) was used as booster. Such a heterologous prime-boost immunization has proven to be more practical than using DNA vaccine or viral-encoded Ag alone (10,11). Moreover, incorporation of cytokine-encoding plasmids in a prime-boost strategy is likely to induce a desirable immune response (12-14).

Of particular interest, mucosal vaccination of heterologous prime-boost regimens could induce effective immune responses at both systemic and mucosal sites, whereas the traditional protocol of prime-boost regimens administered via systemic route provide no mucosal immunity such as mucosal IgA responses (15). However, one important fact is that the order of prime-boost regimens administered via

Correspondence to: Seong-Kug Eo, Department of Microbiology, College of Veterinary Medicine and Bio-Safety Institute of Chonbuk National University, Jeonju 561-756, Korea. (Tel) +82-63-270-3882, (Fax) +82-63-270-3780, (E-mail) vetvirus@chonbuk.ac.kr

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mucosal route should be reversely changed to induce maximal mucosal and systemic immunity (15). Consequently, maximal mucosal and systemic immunity were achieved if recombinant viral vector was used to mucosally prime animals and DNA vaccine was used as a mucosal booster. Here, we decided to assess the reason why the mucosal protocol of prime-boost vaccination differs from that of systemic vaccination. During studying such an issue, we gained the fact that optimally primed immunity could be critically needed to induce maximal immunity following boosting. Thus, this report discussed the important point that should be concerned in mucosal immunization of heterologous prime-boost regimens.

Materials and Methods

Mice and viruses. Female 4- to 5-week old BALB/c (H-2^d) and C57BL/6 (H-2^b) mice were purchased and housed in animal facilities at Chonbuk National University. B cell-deficient (μK/O) mice on the H-2^b background were bred in our pathogen-free facility as previously described (16). The lack of IgM^+ B cells in these mice was confirmed by FACS analysis. Herpes simplex virus type-1 (HSV-1) KOS strain were grown in Vero cells obtained from American Type Culture Collection (Rockville, MD), whereas recombinant vaccinia virus expressing gB (rvacgB) and vaccinia virus tk- (vvtk-) were grown in CV-1 cells (ATCC). The viruses were concentrated, titrated, and stored in aliquots at -80°C until use.

Preparation of plasmid DNA vaccine. Plasmid DNA encoding gB (gB DNA) under the cytomegalovirus promoter has been described in detail elsewhere (17). The plasmid DNA was purified by polyethylene glycol precipitation by the method of Sambrook *et al.* (18) with some modifications. Cellular proteins were precipitated with 1 volume of 7.5 M ammonium acetate followed by isopropanol precipitation of the supernatant. After polyethylene glycol precipitation, plasmids were phenol-chloroform extracted (3×) and precipitated with pure ethanol. The quality of DNA was checked by electrophoresis on 1% agarose gels. The amount of endotoxin was measured by Limulus Amebocyte lysate (LAL) test (<0.05 EU). The effect of endotoxin in vivo was addressed in parallel by administration of control vector.

Immunization and sample collection. Groups of mice (5- to 6-week old female mice) were immunized with either 100μg of gB DNA or 10^6 pfu of rvacgB via the intranasal or intramuscular route, and then boosted 10 days later with alternative vaccine vehicle via same route. Serum samples from mice were collected by retroorbital bleeding. Vaginal lavages were obtained by introduction of 100μl of PBS (pH 7.2) into the vaginal canals, followed by recovering with micropipet.

ELISA for gB-specific antibody. gB-specific antibodies in the samples were determined by standard ELISA as described previously (19). Briefly, ELISA plates were coated with gB protein, and goat-antimouse IgG (Southern Biotechnology Associate Inc. [SBA], Birmingham, AL) or rabbit-antimouse IgA (Zymed, San Francisco, CA), then incubated overnight at 4°C. The plates were then washed with PBST (3×) and blocked with 3% dehydrated milk. Samples were twofold serially diluted, incubated for 2 h at 37°C, and then incubated with goat antimouse IgG-conjugated horseradish peroxidase (IgG-HRP) for 1 h. For measurement of IgA levels in vaginal lavage, biotinylated goat antimouse IgA was first added for 2 h at 37°C followed by peroxidase-conjugated streptavidin (Jackson ImmunoResearch Lab., West Grove). The color was developed by adding the substrate solution (11 mg of 2, 2-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid in 25 ml of 0.1M citric acid, 25 ml of 0.1M sodium phosphate, and 10μl of hydrogen peroxide). Antibody concentrations were calculated with an automated ELISA reader (Spectra MAX340, Molecular Devices, Sunnyvale, CA).

Quantification of cytokine-producing cells. The enzyme-linked immunospot (ELISPOT) assay was used for quantification of cytokine-producing cells as described previously (20). Briefly, ELISPOT plates (Milipore, Molseheim, France) were previously coated with IL-4 or IFN-γ anti-mouse Ab. The immune T cells (responder cells) were mixed with syngeneic splenocytes (stimulator cells) pulsed with UV-inactivated HSV (MOI, 5.0 before UV inactivation) for $CD4^+$ T helper cells, or $gB_{498\text{-}505}$ (SSIEFARL) peptide specific for MHC class I (H-2b)-restricted $CD8^+$ T cells. Coincubation of the responder and stimulator cells was continued for 72 h at 37°C. The ELISPOT plates were washed three times with PBS and three times with PBST, and then biotinylated IL-4 or IFN-γ Ab was added to the plates for 1 h at 37°C. The spot was developed using nitroblue tetrazolium (NBT; Sigma, St. Louis, MO) and 5-bromo-4-chloro-3-indolylphosphate (BCIP; Sigma) as a substrate following incubation with alkaline phosphatase-conjugated streptavidin (Jackson ImmunoResearch) for 1 h and counted 24 h later under a stereomicroscope.

CTL assay. CTL activity was accessed by a standard 5h ^{51}Cr-release assay against labeled target cells as previously described (21). Splenocytes and corresponding DLN lymphocytes (effector cells) were re-stimulated *in vitro* with syngeneic splenocytes pulsed with $gB_{498\text{-}505}$ (SSIEFARL) peptide specific for MHC class I (H-2^b)-restricted $CD8^+$ T lymphocytes at concentration of 10μg/ml for 5 days. The effector

cells were then mixed at various ratios with ^{51}Cr-labelled target cells for 5 h. The target cells included MHC-matched EL-4 (H-2b) and MHC-mismatched EMT-6 (H-2^{d}) pulsed with SSIEFARL peptide. Spontaneous release of ^{51}Cr was determined by incubating the target cells with medium alone, and maximum release was determined by adding Triton X-100 to a final concentration of 5%. To calculate specific lysis of targets, the percent lysis of irrelevant targets was subtracted from the percent lysis of specific targets. The percent of specific lysis was calculated as follows: 100×([experimental release-spontaneous release]/[maximum release-spontaneous release]). Each experiment was performed twice using triplicate samples. *Statistical analysis.* Significant differences between groups were evaluated using the student's *t*-test.

Results

Effect of booster mucosally administered at different time on Ab and CD4^{+} T cells responses. To address the reason why the mucosal protocol of prime-boost vaccination differ from that of systemic vaccination, we first analyzed the appropriate time of boost immunization in mucosal immunization of prime-boost regimens. Groups of mice immunized i.n. with either gB DNA or rvacgB were boosted with alternative vector 10, 20, 40 and 60 days later, then gB-specific serum IgG and vaginal IgA responses were evaluated. As shown in Fig. 1A, gB DNA greatly boosted serum IgG levels induced with rvacgB-priming even 60 days later. Of particular interest, gB DNA-primed serum IgG level increased with administration of rvacgB as booster at 20 day, then declined up to the level of 10-day boosting time. These boosting trends by administration of booster at different times were also evident in vaginal gB-specific IgA levels (Fig. 1B). In particular, vaginal IgA level primed with rvacgB was greatly boosted with same rvacgB 60 days later unlike serum gB-specific IgG data (Fig. 1B).

The effect of booster administration at different times on CD4^{+} T cell-mediated immunity was also evaluated, as judged by enumeration of IFN-γ or IL-4 producing cells (Table I). Similarly, CD4^{+} T cell-mediated immunity primed with rvacgB was boosted with gB DNA given mucosally even 60 days later. However administration of gB DNA as booster at 60 day showed less number of IFN-γ or IL-4 producing cells than those in mice boosted at 20 or 40 day (Table I).

Requirement of optimally primed immunity for the effective boosting of humoral immunity. Mucosal immunization of DNA vaccine at priming provides very weak immunity like primed-naive state, even if mucosal immunization induces systemic and mucosal immunity. Thus it is hypothesized that optimally primed immunity is critically necessary for eliciting effective boosting effect. To prove this hypothesis, we immunized i.n. BALB/c (H-2^{d}) mice with the different doses of gB DNA or coadministration of gB DNA plus cholera toxin B (CTB) as mucosal adjuvant at priming, then i.n. boosted primed mice with rvacgB as booster. gB-specific serum IgG and vaginal IgA

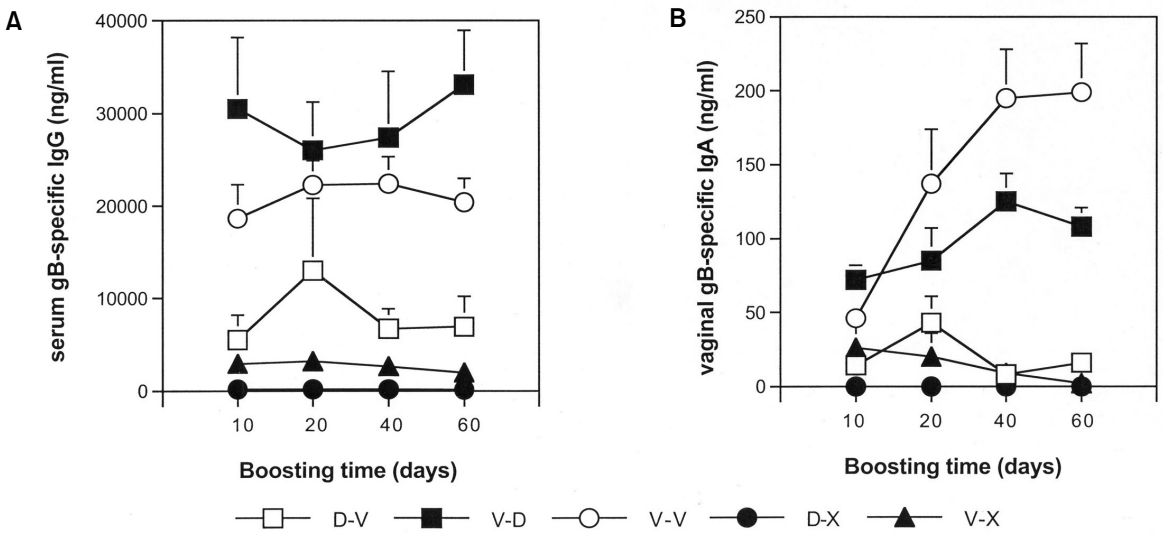

Figure 1. Influence of booster mucosally administered at different time on Ab responses. Groups of BALB/c mice (n=6) immunized i.n. with either gB DNA (D) or rvacgB (V) were boosted with alternative vector 10, 20, 40 and 60 day later. gB-specific serum IgG (A) and vaginal IgA (B) levels were determined by the ELISA on the 10th day post-boosting. The data in figure is showing the average± SD of six mice per group at each boosting time.

Table I. Effect of booster mucosally administered at different time on the frequency of cytokine-producing $CD4^+$ T cells[a]

Regimens	Spleen						Cervical LN					
	d20		d40		d60		d20		d40		d60	
	IFN-γ	IL-4	IFN-γ	IL-4	IFN-γ	IL-4	IFN-γ	IL-4	IFN-γ	IL-4	IFN-γ	IL-4
D-D	11±3	3±4	13±10	-	-	-	-	1±3	-	-	2±3	-
D-V	64±37	48±30	79±31	42±17	54±30	21±10	44±20	28±15	29±11	21±14	20±9	15±10
V-D	113±49	71±28	131±35	72±24	75±25	31±10	84±29	47±19	73±27	40±16	39±22	34±17
V-V	92±47	39±21	72±45	40±8	58±27	13±7	44±21	37±14	51±26	31±12	14±4	8±7

[a]Groups of BALB/c mice immunized i.n. with either gB DNA (D) or rvacgB (V) were boosted with alternative vector 20, 40, and 60 days later. Following boosting, mice were killed on the 15th day, and spleen and cervical LN were collected to enumerate IFN-γ and IL-4-producing $CD4^+$ T cells by the ELISPOT approach. Results are the mean± SD of four mice per group.

level of mice immunized with such protocols were compared to those of mice immunized with rvacgB prime-gB DNA boost regimen. As shown in Fig. 2A, increase of gB DNA dose or co-administration with CTB at mucosal priming provided enhanced serum gB-specific IgG level but still significantly less than rvacgB prime-gB DNA boost immunization (P=0.045). Similarly, increase of gB DNA dose or incorporation

Figure 2. Influence of primary immunity primed with different doses of DNA vaccine or incorporation of adjuvant on the boosting effect of recombinant viral vector. Groups of BALB/c ($H\text{-}2^d$) mice were immunized i.n. with the different doses (100μg or 300μg) of gB DNA or incorporation of cholera toxin B (CTB) and boosted via the same route with rvacgB 10 days later. gB-specific serum IgG (A) and vaginal IgA (B) levels were determined by the ELISA on the 10th day post-boosting. The data in figures represent the average±SD of seven mice per group. The statistical values (p-value) represent the difference of each group from rvacgB primed/gB DNA boosted group (V-D100).

with CTB at mucosal priming also induced enhanced vaginal gB-specific IgA level (Fig. 2B). Interestingly, unlike serum gB-specific IgG level, the mucosal coadministration of gB DNA plus CTB at priming significantly provided comparable vaginal IgA level to rvacgB prime-gB DNA boost immunization (P=0.329) (Fig. 2B).

Effect of humoral pre-existing immunity on CD8$^+$ T cell-mediated responses induced by prime-boost regimens. An analysis of the pattern of CD8$^+$ T cell responses in C57BL/6 (H-2^b) mice immunized by heterologous prime-boost regimens revealed similar results as those observed with CD4$^+$ T cell and Ab responses (15). To prove how mucosal or systemic immunization of prime-boost regimens elicited different outcomes in CD8$^+$ T cell-mediated immunity, we addressed the following possibility. Immunity primed with gB DNA or rvacgB could interfere boosting effect of vaccinia viral vector as booster. Especially, when rvacgB as booster is administered mucosally, the gB- or vaccinia viral vector-specific mucosal Ab produced by gB DNA or rvacgB at priming could provide barrier immunity to protect invasion of virus as booster. To address this possibility, we used B cell-deficienct (μK/O) mice (H-2^b) immunized i.n. or i.m. with prime-boost regimens, then CTL activity and IFN-γ producing CD8$^+$ T cell responses were measured. These mice produce no Abs at mucosal sites to interfere boosting effect of rvacgB as booster. As shown in Fig. 3AB, rvacgB prime-gB DNA boost protocol still provided the best CD8$^+$ T cell-mediated immunity in mucosal immunization. This pattern is same as shown in wt mice (data not shown). Interestingly, the potency of CD8$^+$ T cell-mediated immunity induced by gB DNA systemic prime-rvacgB systemic boost was more evident in spleen of μK/O mice (Fig. 3A). IFN-γ producing CD8$^+$ T cell response also showed the same pattern (Fig. 4). This result indicates that both serum and mucosal Abs produced at priming provide no interference to boosting effect of rvacgB as booster.

Influence of priming doses on the boosting of CD8$^+$ T cell-mediated immunity. We also addressed the effect of gB DNA priming dose on CD8$^+$ T cell-mediated CTL activity. We immunized i.n. C57BL/6 mice with the different doses of gB DNA at priming, then boosted primed mice via the same route with rvacgB. The CTL activity of mice immunized with such protocols were compared to those of mice immunized with rvacgB prime-gB DNA boost regimen. The mucosal administration of 300μg gB DNA at priming induced comparable CTL activity following mucosal boosting with rvacgB to rvacgB mucosal prime-gB DNA mucosal boost immunization (Fig. 5).

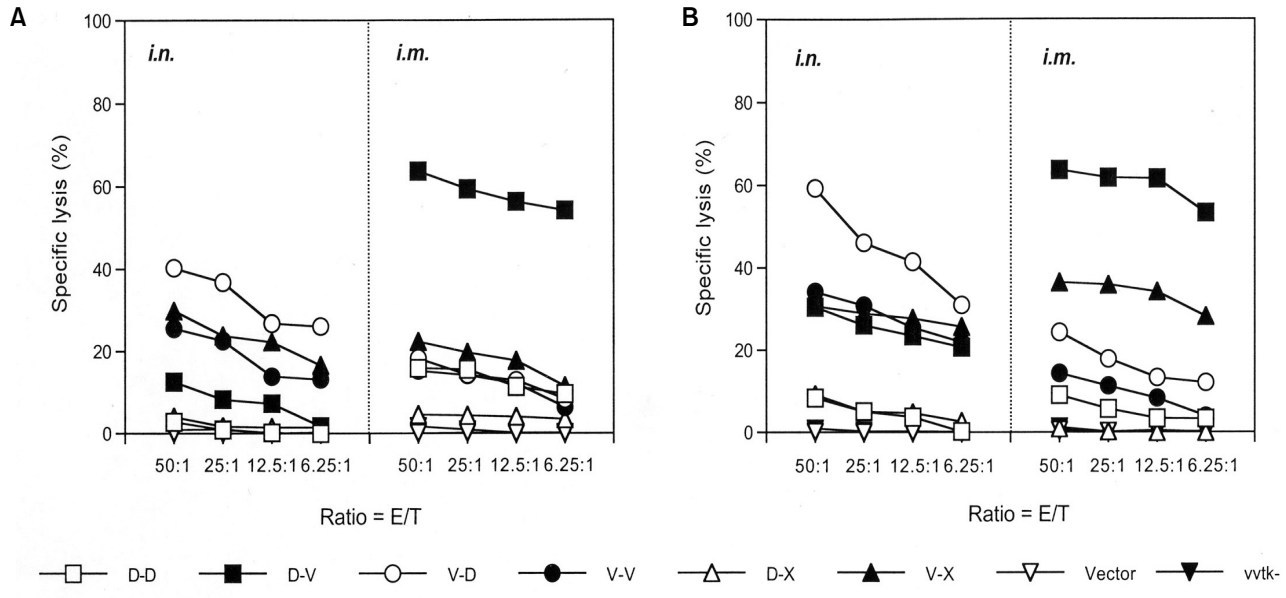

Figure 3. CD8$^+$ T cell-mediated immunity in B cell-deficient (μK/O) mice immunized with heterologous prime-boost regimens. Groups of μK/O mice (H-2^b) were immunized mucosally (i.n.) or systemically (i.m.) with gB DNA (D) or rvacgB (V) and boosted via the same route used for priming with the alternative vaccine type 10 days later. Two weeks later, spleen (A) and draining LN (i.e., cervical LN for mucosal immunization and popliteal plus inguinal LN for systemic immunization) (B) were collected to measure CD8$^+$ T cell-mediated immunity. CD8$^+$ T cell-mediated immunity was evaluated by CTL assay after restimulating with MHC class I-restricted (H-2^b) peptide (SSIEFARL) for 5 days. The data in figures represent the average±SD of four mice per group.

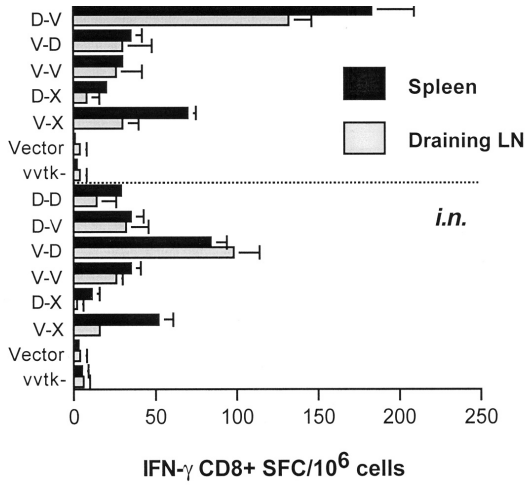

Figure 4. Frequency of IFN-γ-producing $CD8^+$ T cells in B cell-deficient (μK/O) mice immunized with heterologous prime-boost regimens. Groups of μK/O mice were immunized as described in *Materials and Methods*. The number of IFN-γ -producing $CD8^+$ T cells was determined by ELISPOT assay after restimulation with MHC class I-restricted ($H\text{-}2^b$) peptide (SSIEFARL) for 5 days. The data in figures represent the average ±SD of four mice per group.

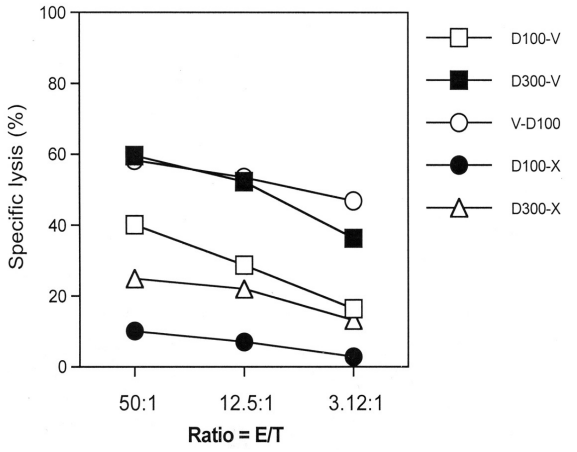

Figure 5. Influence of primary immunity primed with different doses of DNA vaccine on the boosting effect of $CD8^+$ T cell-mediated immunity. Groups of C57BL/6 (H-2b) mice were immunized i.n. with different doses (100μg or 300μg) of gB DNA and boosted via the same route with rvacgB 10 days later. $CD8^+$ T cell-mediated immunity was evaluated by the CTL assay as described in *Materials and Methods*. The data in figure represent the average of four mice per group.

These results imply that the effective boosting immunization could need optimal immunity induced at priming step if administered mucosally.

Discussion

The present report tried to assess the importance of primary responses induced at priming step in mucosal immunization of heterologous prime-boost regimens. Immunity primed with recombinant viral vector was effectively boosted with DNA vaccine even 60 days later. In particular, it is interesting that optimal primary immunity primed by increasing doses or incorporating an adjuvant could need to induce the effective boosting of both humoral and cell-mediated immunity. Humoral pre-existing immunity induced at priming step was also unlikely to interfere the boosting effect of $CD8^+$ T cell-mediated immunity by recombinant vaccinia virus vaccine, as evident in experiment using B cell- deficient (μK/O) mice. Therefore, this paper provides the fact that the optimal primary responses should be considered in mucosal immunization of heterologous prime-boost regimens for inducing optimal immunity at both mucosal and systemic sites.

The discovery that plasmid DNA encoding viral proteins could be used for protection against viral infection opened a new era of vaccination strategy (1). However, DNA vaccines are yet distant from the practical use because of its weak immunogenicity (1-4). In recent, the usefulness of DNA vaccines at priming step of heterlogous prime-boost vaccination have move to DNA vaccines closer to practical reality (10,11,23,24). In particular, it was interesting that mucosal vaccination of heterologous prime-boost regimens induced maximal humoral and cell-mediated immunity at both mucosal and systemic sites if administered in contrast with the protocol of systemic immunization (15). Mucosal surfaces represent the primary site for the transmission of several pathogenic viruses including human immunodeficiency virus and herpes simplex virus. In consequence, the data presented here provide the strategy clue for inducing effective mucosal barrier against viral infection. Firstly, the priming step seems be the critical event for optimal mucosal immunity. This expectation is on same line with previous report (15), which showing that priming mucosally is critical when prime-boost protocols were used in heterogenous route (systemic or mucosal route). Especially, naked DNA vaccine administered mucosally is inferior inducer for vaginal IgA to recombinant viral vector. Such a potency gap could be narrowed by either increasing priming dose or incorporating a mucosal adjuvant. These facts imply that enough primary responses over threshold for getting maximal boosted response could be necessary. In other words, the amount (capacity) and nature (Th1/Th2-type) of primary response induced at priming step could

decide those of the final immunity caused by administration of booster (12,13,25).

Secondly, the reason why recombinant viral vector priming via mucosal route proved superior to DNA vaccine remains to be defined. As one explanation, we tried to assess the potency pattern of $CD8^+$ T cell-mediated response when heterologous prime-boost regimens were mucosally administered into B cell-deficient (μK/O) mice. DNA vaccines appear unaffected by pre-existing immunity because of inert immunogen itself (26,27). Thus, this makes them potentially valuable vaccines in newborn animal that possess high levels of passive immunity (27,28). In contrast, recombinant viral vector used as booster can be affected by pre-existing immunity (26-28). Thus, it is hypothesized that immunity induced at priming step could interfere boosting effect of recombinant vaccinia virus vaccine. Unexpectedly, humoral pre-existing immunity induced at priming step did not suppress the boosting effect of recombinant vaccinia virus vaccine as booster. Although this concept should be further defined, B cells and Abs are unlikely to interfere the role of recombinant viral vector as booster for $CD8^+$ T cell-mediated responses.

In case of that heterologous prime-boost regimens were administered systemically, it is guessed that the critical event in priming depends on the quality of immunogen (DNA vaccine better than vaccinia virus vaccine) and that boosting is more dependent on the quantity of immunogen expressed (vaccinia virus vaccine better than DNA vaccine) (29). However, there are data in mice (30) and some data in humans (26) suggesting that pre-existing vaccinia immunity, such as that occurring in a large proportion of the adult population because of smallpox vaccination, limits the effectiveness of recombinant vaccinia vectors as vaccines. As bypass to solve this dilemma, mucosal vaccination has been suggested because the induction sites of mucosal immune system may still nave to the vaccinia antigen (31). Therefore, mucosal administration of heterologous prime-boost regimens is the effective approach to induce wide and high immunity. Consequently, we discussed the importance of primary immunity that could help to make the optimal strategy in such a mucosal prime-boost vaccination.

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