

Modulation of protective immunity against herpes simplex virus via mucosal genetic co-transfer of DNA vaccine with β_2 -adrenergic agonist

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Abbreviations: ABTS, 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid; APC, antigen-presenting cell; CT, cholera toxin; DC, dendritic cell; DP, Depo-Provera; GAP, GTPase activating protein; gB, glycoprotein B; HIV, human immunodeficiency virus; HSV, herpes simplex virus; i.m., intramuscular; i.n., intranasal; LN, lymph node; MOI, multiplicity of infection

Abstract

Cholera toxin, which has been frequently used as mucosal adjuvant, leads to an irreversible activation of adenyl cyclase, thereby accumulating cAMP in target cells. Here, it was assumed that β_2 -adrenergic agonist salbutamol may have modulatory functions of immunity induced by DNA vaccine, since β_2 -adrenergic agonists induce a temporary cAMP accumulation. To test this assumption, the present study evaluated the modulatory functions of salbutamol co-administered with DNA vaccine expressing gB of herpes simplex virus (HSV) via intranasal (i.n.) route. We found that the i.n. co-administration of salbutamol enhanced gB-specific IgG and IgA responses in both systemic and mu-

cosal tissues, but optimal dosages of co-administered salbutamol were required to induce maximal immune responses. Moreover, the mucosal co-delivery of salbutamol with HSV DNA vaccine induced Th2-biased immunity against HSV antigen, as evidenced by IgG isotypes and Th1/Th2-type cytokine production. The enhanced immune responses caused by co-administration of salbutamol provided effective and rapid responses to HSV mucosal challenge, thereby conferring prolonged survival and reduced inflammation against viral infection. Therefore, these results suggest that salbutamol may be an attractive adjuvant for mucosal genetic transfer of DNA vaccine.

Keywords: albuterol; herpes simplex virus vaccines; immunity, mucosal; Th1 cells; Th2 cells; vaccine, DNA

Introduction

Immunization with naked DNA encoding various genes has proven to be a valuable means of immunity induction (Donnelly *et al.*, 1997). The great majority of DNA vaccine studies have used the intramuscular (i.m.) or gene gun delivery approaches for the administration of DNA vaccine, and this method has produced the best results. Protective immune responses after DNA vaccination have been demonstrated against several diseases and pathogens using different immunization routes including i.m., intravenous (i.v.), oral, intranasal (i.n.), and vaginal administration in both experimental animals and humans (Klavinskis *et al.*, 1999; Gautam *et al.*, 2000; Heckert *et al.*, 2002). Among different strategies for genetic immunization, immunity induction at mucosal sites is important in vaccine development since mucosal surfaces are the primary site for transmission of several viruses, including human immunodeficiency virus (HIV) and herpes simplex virus (HSV) (Ogra, 1999; Pope and Haase, 2003). However, DNA vaccination through mucosal route has typically failed to induce effective immune responses at mucosal sites (Kuklin *et al.*, 1997; MacLaughlin *et al.*, 1998). In recent, interests have focused on the co-expression of cytokines and chemokines to modulate immunity induced by DNA vaccine (Eo *et al.*, 2001a; 2001b; Fukuiwa *et al.*, 2008). Accor-

dingly, both molecule types are involved in regulating the inflammatory reaction and the subsequent adaptive Th1 or Th2-type of T-cell reaction that occurs in draining lymph nodes (LN). However, the co-expression of such cytokines and chemokines delivered through mucosal routes might provide sub-optimal protection against pathogenic challenge.

Cholera toxin (CT) is a mucosal adjuvant frequently used in the development of mucosal immune responses (Freytag and Clements, 2005). CT loses its adjuvant and toxic properties upon deactivation of the ADP-ribosylation function of its subunit A (Elson and Ealding, 1984; Lycke, 1996). The ADP-ribosylation of the Gs protein typically leads to an irreversible activation of adenylyl cyclase and subsequent accumulation of cAMP. Additionally, some of the effects of CT on immunocytes can be reproduced by cAMP analogues or adenylyl cyclase stimulators (Lycke, 1993; Leal-Berumen *et al.*, 1996; Agren *et al.*, 1997). This suggested that β_2 -adrenergic agonists may have modulatory functions of immunity induced by DNA vaccines, since such β_2 -adrenergic agonists, anti-asthmatic drugs, induce temporary cAMP accu-

mulation in its target cells (Strosberg, 1997). Here, we assessed the modulatory effects of salbutamol co-administered i.n. with DNA vaccine against HSV. The co-administration of salbutamol was observed to induce the enhanced immune responses at both systemic and mucosal tissues, but optimal dosages of co-administered salbutamol should be carefully evaluated to induce the effective protection against HSV infection.

Results

Modulation of systemic and mucosal antibody responses via co-transfer of β_2 -adrenergic agonist

To assess the immunomodulatory functions of β_2 -adrenergic agonists as mucosal adjuvant, we co-immunized mice with HSV pCIgB DNA vaccine in the presence of salbutamol. The immunization was i.n. performed by depositing several different mixtures of pCIgB DNA vaccine dissolved in salbutamol-containing PBS onto the nares of deeply anesthetized mice three times at 7-day intervals. The gB-specific IgG levels in sera were

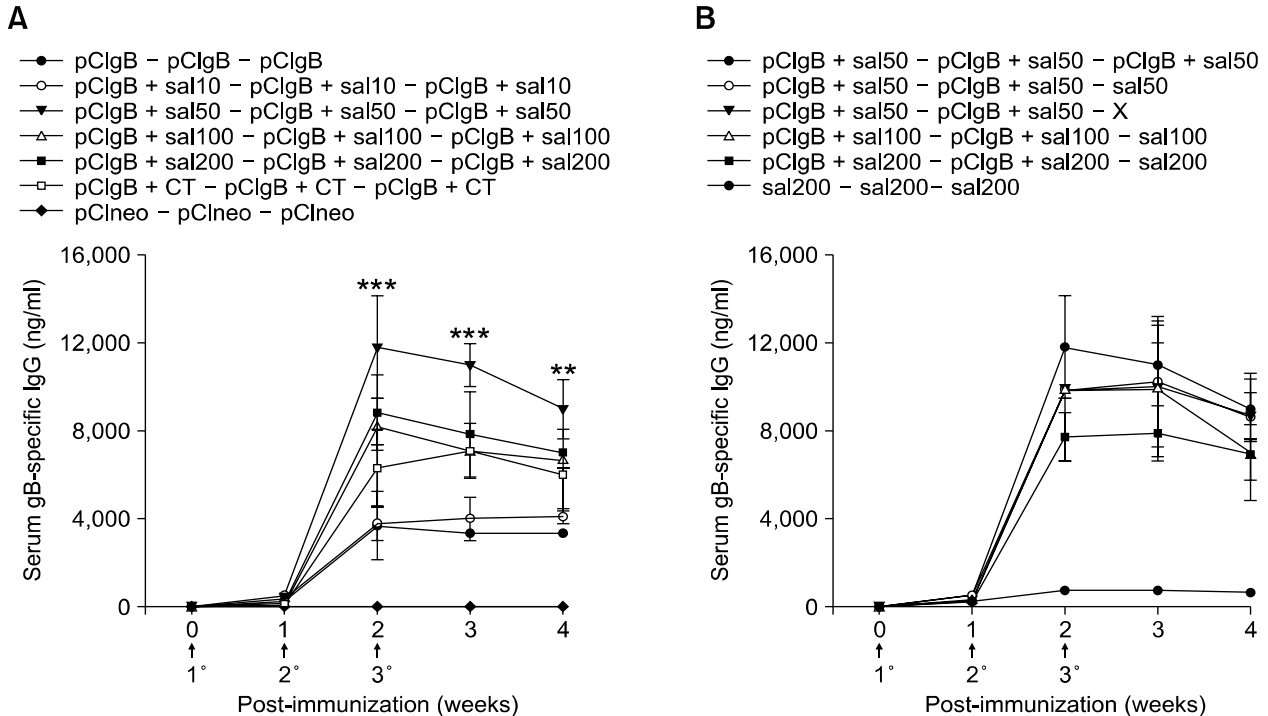


Figure 1. Serum gB-specific IgG levels of animals immunized with pCIgB in the presence of salbutamol. (A and B) Groups of BALB/c (H-2^d) were co-immunized i.n. with the indicated mixture of pCIgB DNA vaccine (100 μ g) and salbutamol (10, 50, 100, and 200 μ g) three times at 7-day intervals. Some mice were immunized i.n. with control vector (pCI-neo) to remove the effect of the plasmid backbone. The gB-specific IgG levels in sera were determined by conventional ELISA on the seventh day after each immunization. Arrows in graph denote date to immunize mice: 1°, first immunization; 2°, second immunization; 3°, third immunization. The graph represents the average \pm SD of values obtained from seven mice per group. ** $P < 0.01$; *** $P < 0.001$ when compared to pCIgB-immunized group.

then determined on the seventh day after each immunization, as shown in Figure 1A. The control vector (pCI-neo) induced no significant gB-specific IgG responses, whereas pCIgB DNA vaccine administered via i.n. route produced detectable IgG levels after the first immunization and such IgG levels were enhanced by a subsequent second administration. However, the gB-specific IgG levels were not significantly increased by a third administration, suggesting that gB-specific IgG responses might be saturated by two i.n. administrations of pCIgB DNA vaccine. In addition, the levels of gB-specific IgG in sera were enhanced by co-administration of salbutamol. In particular, groups inoculated with the mixture of pCIgB

plus 50 µg salbutamol displayed higher enhancement of gB-specific IgG levels compared to groups that received the co-administration of 100 or 200 µg salbutamol (Figure 1A). Moreover, the enhanced responses of gB-specific IgG in mice that were co-inoculated with 50 µg salbutamol were more apparent than those in group co-administered with CT. Such enhancement of IgG responses was marginally declined by subsequent third co-administration of salbutamol. Also, mice that received two times of pCIgB plus salbutamol (50 µg) mixture elicited comparable levels of gB-specific IgG to those of mice given three times of same mixture (Figure 1B). These results suggest that salbutamol has immunomodulatory func-

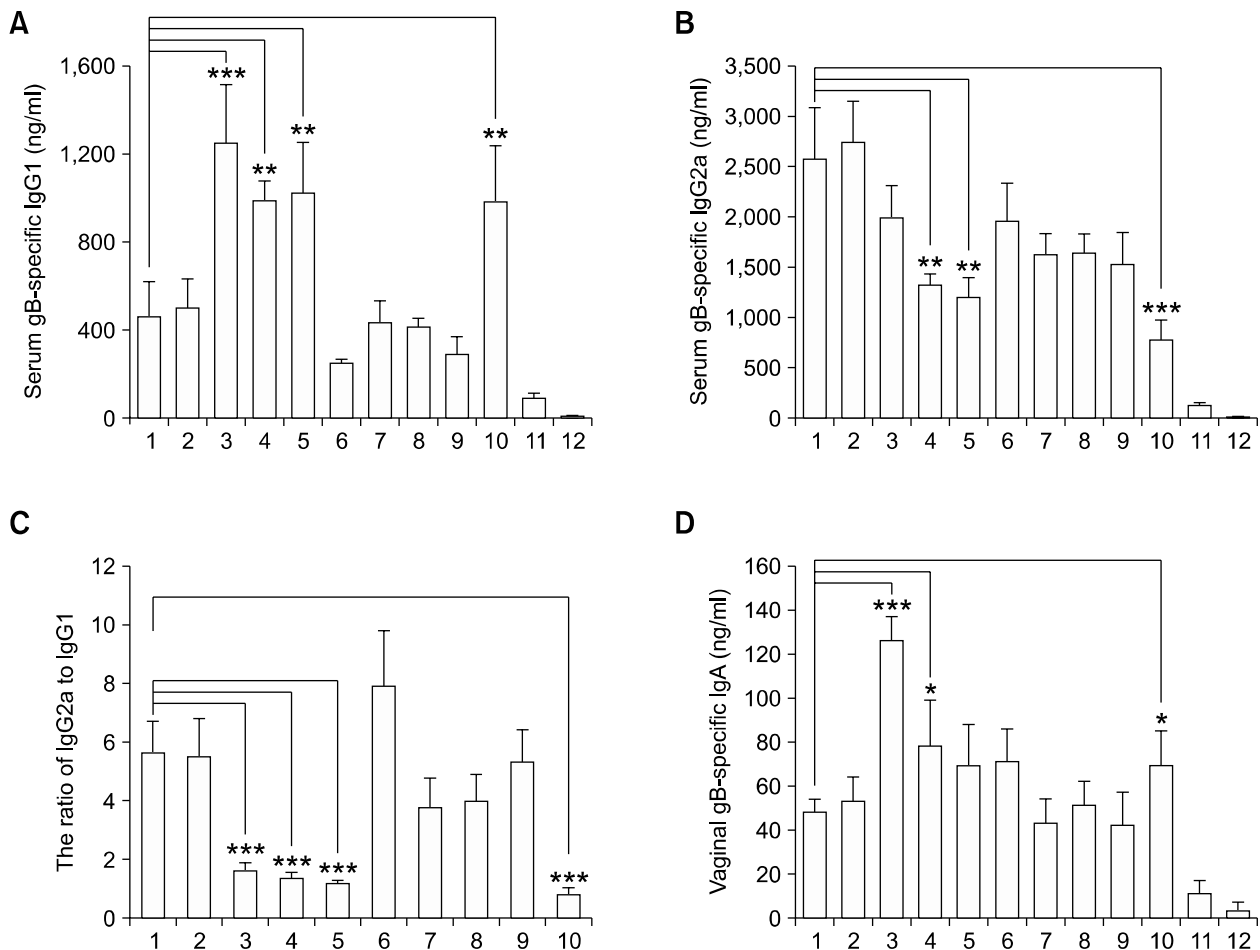


Figure 2. Distribution of serum gB-specific IgG isotypes (IgG1 and IgG2a) and vaginal gB-specific IgA in mice co-immunized i.n. with pCIgB and salbutamol. Groups of BALB/c (H-2^d) mice were co-immunized i.n. with the indicated mixture of pCIgB DNA vaccine (100 µg) and salbutamol (10, 50, 100, and 200 µg) three times at 7-day intervals. Some mice were immunized i.n. with control vector (pCI-neo) to remove the effect of the plasmid backbone. Seven days following the final immunization, the gB-specific IgG isotypes (A) IgG1, (B) IgG2a, (C) the ratio of IgG2a to IgG1 in the sera, and (D) vaginal IgA were determined by conventional ELISA. The bars represent the average ± SD of values obtained from seven mice per group. 1, pCIgB - pCIgB; 2, pCIgB + sal10 - pCIgB + sal10 - pCIgB + sal10; 3, pCIgB + sal50 - pCIgB + sal50 - pCIgB + sal50; 4, pCIgB + sal50 - pCIgB + sal50 - sal50; 5, pCIgB + sal50 - pCIgB + sal50 - X; 6, pCIgB + sal100 - pCIgB + sal100 - pCIgB + sal100; 7, pCIgB + sal100 - pCIgB + sal100 - sal100; 8, pCIgB + sal200 - pCIgB + sal200 - pCIgB + sal200; 9, pCIgB + sal200 - pCIgB + sal200 - sal200; 10, pCIgB + CT - pCIgB + CT - pCIgB + CT; 11, sal200 - sal200 - sal200; 12, pCIneo - pCIneo - pCIneo. **P* < 0.05; ***P* < 0.01; ****P* < 0.001 when compared between the indicated groups.

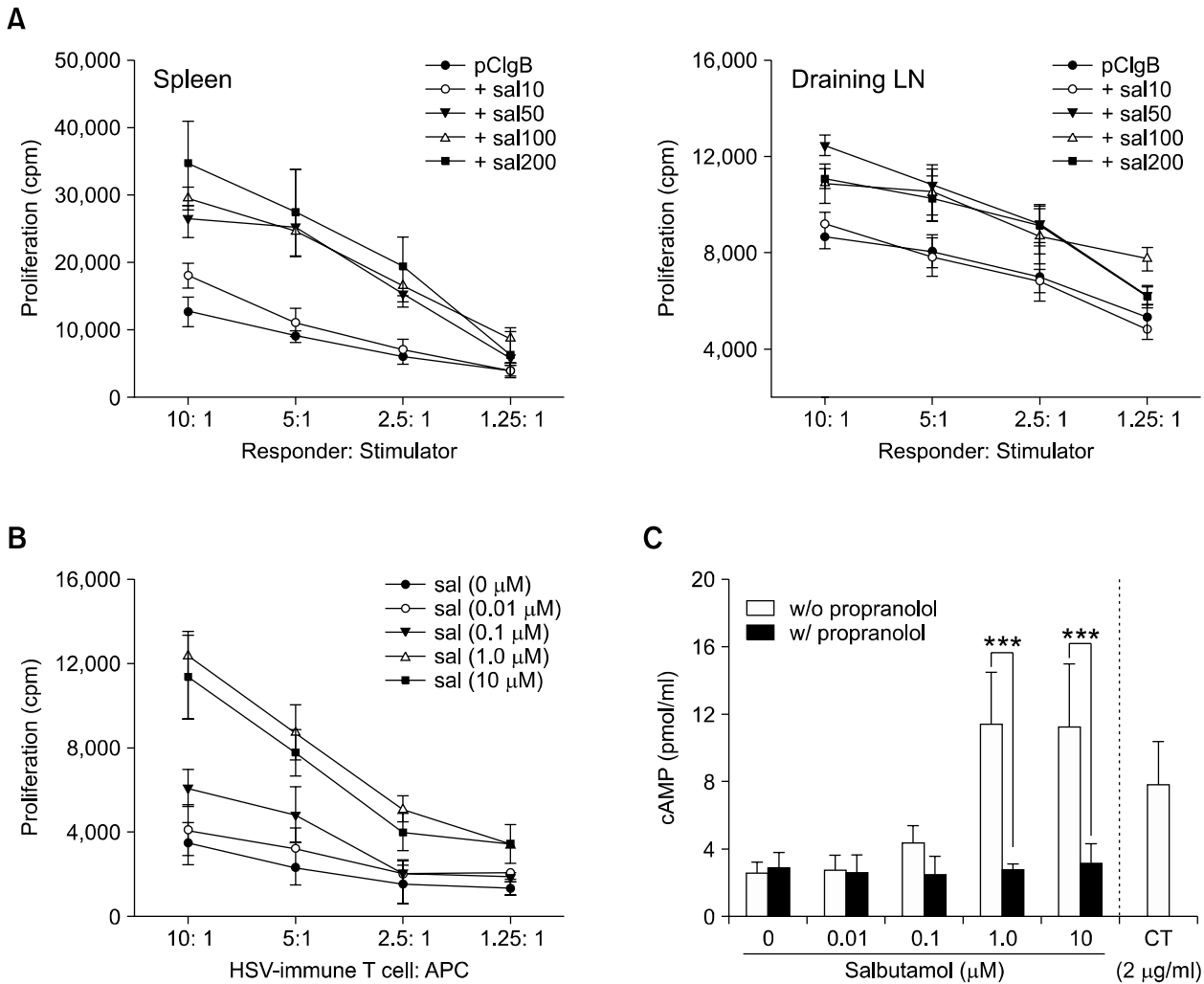


Figure 3. HSV-specific proliferation of splenocytes and LN cells obtained from pClgB-immunized mice and cAMP production by salbutamol. (A) Proliferation of splenocytes and LN cells after specific restimulation with HSV antigen. Groups of BALB/c ($H-2^d$) mice were co-immunized i.n. with a mixture of pClgB DNA vaccine (100 μ g) and salbutamol (10, 50, 100, and 200 μ g) three times at 7-day intervals. Two weeks following the final immunization, the responder cells were prepared from spleen and draining LN, and mixed with irradiated syngeneic-enriched APCs that had been pulsed with UV-inactivated HSV. (B) Increased HSV-specific proliferation of $CD4^+$ T cells in the presence of salbutamol. $CD4^+$ T cells purified from HSV-immunized mice were mixed with HSV antigen-pulsed APCs in the presence of the indicated salbutamol concentrations. Following 3-day incubation, the proliferated cells were determined by [3H] thymidine incorporation. The graph represents the average \pm SD of values obtained from three or four individual experiments. (C) The production of cAMP in dendritic cells treated with salbutamol. DC2.4 cells were treated with the indicated concentration of salbutamol in presence or absence of propranolol (β -adrenergic antagonist) and used for determination of intracellular cAMP levels. CT (cholera toxin) was included for positive control. The bars represent the average \pm SD of values obtained from quadruplicate wells. *** $P < 0.001$ when compared between the indicated groups.

tion of DNA vaccine administered via i.n. route, and this immunomodulatory effect was dependent on optimal dose of co-administered salbutamol.

When the modulatory effect of salbutamol on the production of gB-specific IgG isotypes (IgG1 and IgG2a) induced by pClgB was evaluated, mice inoculated with pClgB plus 50 μ g salbutamol mixture had greater production of gB-specific IgG1 isotype and no significant change of IgG2a production (Figure 2A and B). Therefore, the enhancement of IgG1 isotype production through co-administration of salbutamol resulted in a lower ratio

of IgG2a to IgG1 (Figure 2C), which indicates that the co-administration of salbutamol with pClgB DNA vaccine drove T_H2 -biased humoral immunity. Also, group given co-administration of pClgB plus CT mixture showed enhanced production of gB-specific IgG1 isotype which resulted in T_H2 -biased humoral immunity. Similarly, when gB-specific IgA responses at mucosal sites were analyzed on the seventh day after the final immunization, mice that received the mixture of pClgB plus 50 μ g salbutamol had higher IgA levels in the vaginal tract than other groups (Figure 2D). Therefore, these

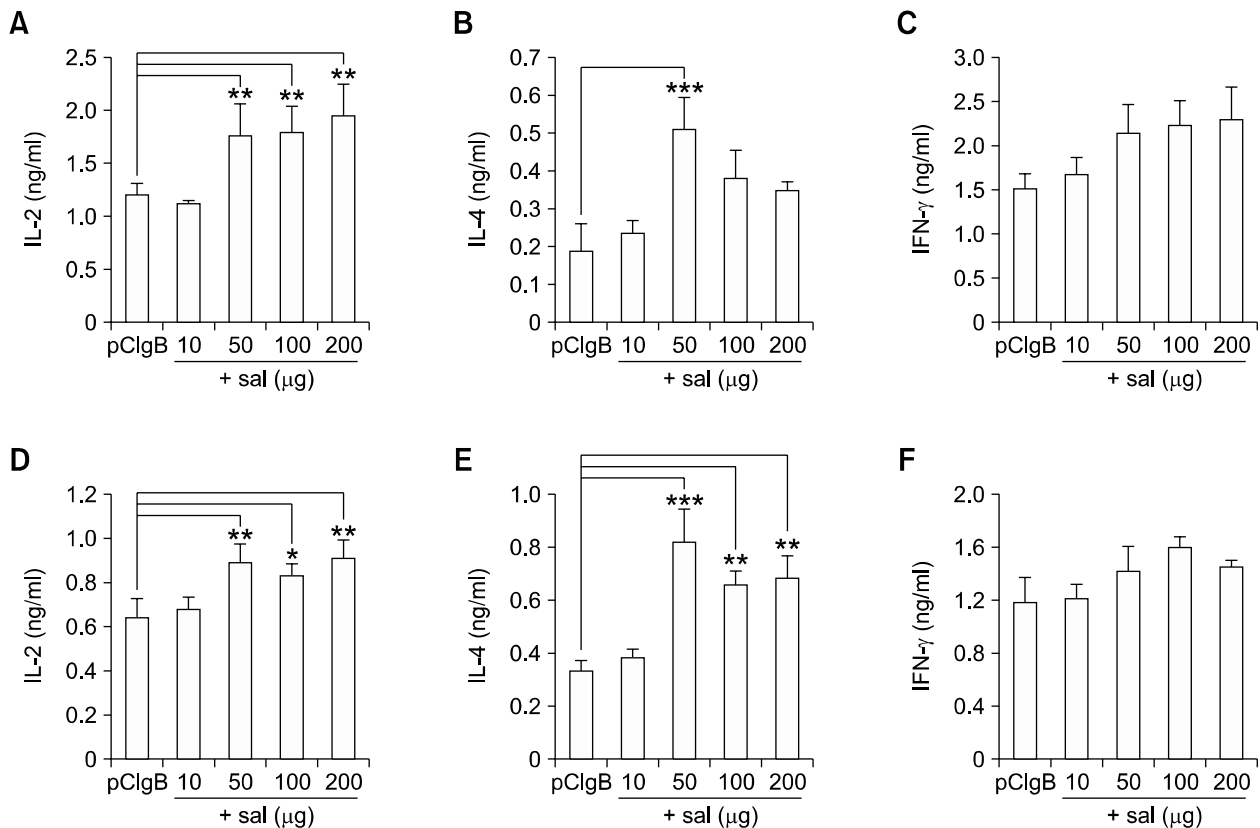


Figure 4. The profile of cytokine production (IL-2, IL-4, and IFN- γ) by the splenocytes and draining LN cells of immunized mice after specific restimulation with HSV antigen. Groups of BALB/c (H-2^d) mice were immunized i.n. with a mixture of pClgB DNA vaccine (100 μ g) and salbutamol (10, 50, 100, and 200 μ g) three times at 7-day intervals. Two weeks following the final immunization, the responder cells were prepared from spleen (A, B, and C) and draining LN (D, E, and F), and mixed with irradiated syngeneic-enriched APCs that had been pulsed with UV-inactivated HSV. Following 3-day incubation, the levels of cytokines in culture supernatants were determined by cytokine ELISA. The bars represent the average \pm SD of values obtained from three or four individual experiments. * P < 0.05; ** P < 0.01; *** P < 0.001 when compared between the indicated groups.

results indicate that salbutamol co-administered via i.n. route modulated gB-specific IgG and IgA responses in both systemic and mucosal tissues, and optimal doses of salbutamol were important for induction of maximal immune responses.

Augmentation of T cell-mediated immunity via co-transfer of β_2 -adrenergic agonist

To further evaluate the modulatory functions of salbutamol co-administered with pClgB DNA vaccine, the effect of co-administered salbutamol on Th-cell proliferative responses and the production of Th1- or Th2-type cytokines in spleen and cervical draining LNs were examined following *in vitro* restimulation with UV-inactivated HSV antigen, which is known to induce the predominant expansion of immune CD4⁺ T cells (Eo *et al.*, 2001a). As shown in Figure 3A, the co-administration of salbutamol (> 50 μ g/mouse) significantly increased the HSV-specific proliferative responses. However, mice that received the co-

administration of 10 μ g salbutamol showed slightly increased proliferation of CD4⁺ Th cells following *in vitro* restimulation with HSV antigen. To further characterize the enhancement of CD4⁺ proliferation by salbutamol, we purified CD4⁺ T cells from HSV-immunized mice and then used for restimulation with HSV antigen in the presence of different concentrations of salbutamol. Salbutamol induced the enhancement of CD4⁺ T cell proliferation by restimulation of HSV antigen in dose-dependent manner (Figure 3B). Also, the presence of salbutamol elicited the accumulation of cAMP in dendritic cells (DC2.4) which could be blocked by the β -adrenergic antagonist (propranolol) (Figure 3C). Therefore, these results indicate that salbutamol could enhance the responses of CD4⁺ Th cells and this enhancement might be mediated by accumulated cAMP.

With regard to cytokine production by restimulated CD4⁺ Th cells, Th1- (IL-2 and IFN- γ) and Th2- (IL-4) type cytokines were significantly produced from CD4⁺ T cells stimulated with HSV antigen

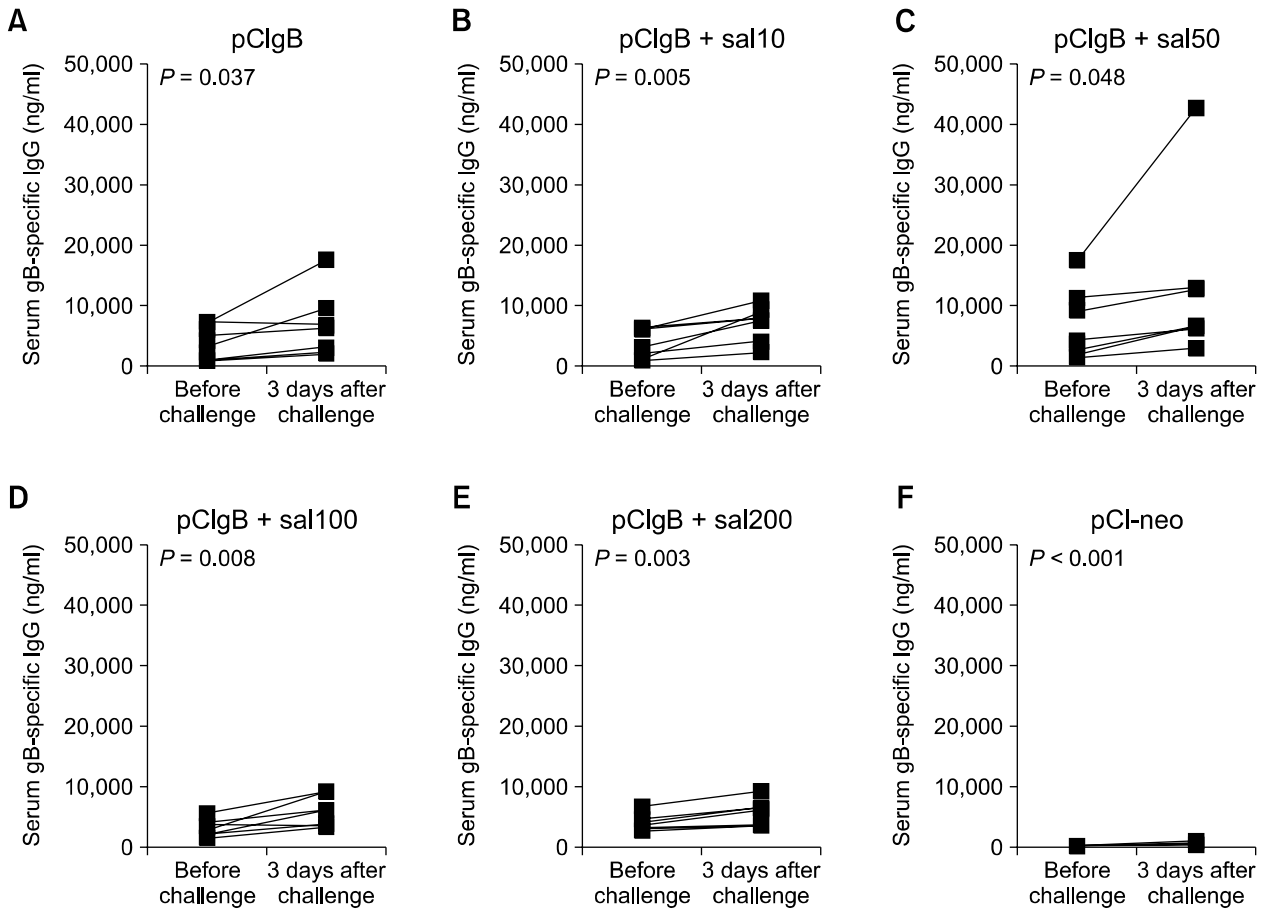


Figure 5. Anamnestic gB-specific IgG responses after HSV challenge. Groups of BALB/c (H-2^d) mice were co-immunized i.n. with a mixture of pCIgB DNA vaccine (100 μ g) and salbutamol (10, 50, 100, and 200 μ g) three times at 7-day intervals. After 2-weeks of the final immunization, the mice were challenged intravaginally with HSV McKrae strain (10^6 PFU). The sera were collected 3 days after challenge and then used for the determination of gB-specific IgG levels by conventional ELISA. The closed square on the graph indicate the individual serum IgG levels of mice before challenge and 3 days post-challenge. *P*-values in graphs were calculated by Student's *t*-test.

following i.n. administration of pCIgB DNA vaccine (Figure 4). The co-administration of salbutamol resulted in significant changes of such Th1- and Th2-type cytokine production from splenocytes and draining LN cells of pCIgB-immunized mice following restimulation with HSV antigen. As shown in Figure 4, the co-administration of salbutamol showed significantly increased production of IL-2 from restimulated splenocytes and draining LN cells, whereas the production of IFN- γ was marginally increased by co-administration of salbutamol. Moreover, it was interesting that the co-administration of 50 μ g salbutamol showed higher production of Th2-type cytokine (IL-4) from splenocytes and draining LN cells than other groups given higher doses (100 and 200 μ g) of salbutamol (Figure 4B and D). Therefore, these results, together with gB-specific IgG isotypes, indicate that mucosal co-delivery of salbutamol with pCIgB DNA vaccine induces Th2-biased immunity against HSV antigen.

Anamnestic response against HSV challenge

To determine if the co-administration of salbutamol with pCIgB DNA vaccine affects the protective responses against mucosal challenge of HSV, groups of mice inoculated with a mixture of pCIgB DNA vaccine plus salbutamol were challenged intravaginally with the virulent HSV-1 McKrae strain (10^6 PFU) 2 weeks after the final immunization. When anamnestic levels of serum gB-specific IgG were initially evaluated 3 days after the challenge, there were no detectable gB-specific IgG responses in mice that were inoculated with the control vector. In contrast, mice that were inoculated with pCIgB DNA vaccine alone displayed IgG levels that were significantly increased by mucosal challenge of HSV (*P* = 0.037) (Figure 5). Furthermore, groups of mice that received a mixture of pCIgB DNA vaccine plus salbutamol showed enhanced levels of anamnestic IgG in response to

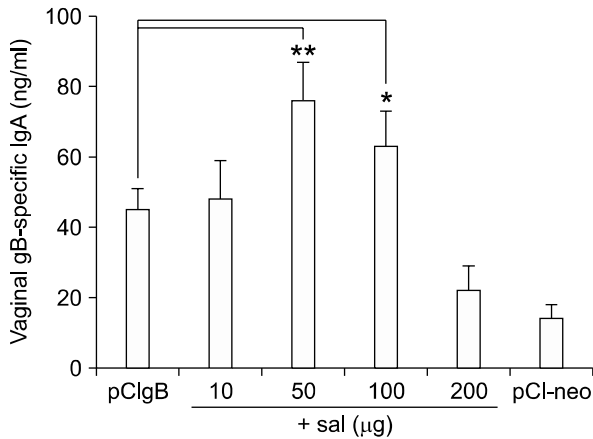


Figure 6. Mucosal gB-specific IgA levels in mice challenged with HSV. Groups of BALB/c (H-2^d) mice were co-immunized i.n. with a mixture of pCIgB DNA vaccine (100 µg) and salbutamol (10, 50, 100, and 200 µg) three times at 7-day intervals. After 2-weeks of the final immunization, the mice were challenged intravaginally with HSV McKrae strain (10⁶ PFU). The vaginal lavage fluids were collected 3 days after challenge and then used for the determination of vaginal gB-specific IgA levels by conventional ELISA. The bars represent the average ± SD of values obtained from seven mice per group. **P* < 0.05; ***P* < 0.01 when compared between the indicated groups.

HSV challenge. In particular, mice that received pCIgB DNA vaccine dissolved in 50 µg salbutamol showed markedly enhanced levels of anamnestic

IgG responses, with IgG level being increased by up to 2 to 3-fold in some mice (*P* = 0.048) (Figure 5). Also, when vaginal IgA levels in mice co-immunized with salbutamol were determined 3 days after the HSV challenge, similar anamnestic trends were shown by markedly enhanced levels of vaginal IgA in mice that received a mixture of pCIgB DNA vaccine plus 50 µg salbutamol (Figure 6). These results indicate that the co-administration of salbutamol at optimal doses could provide effective and rapid responses against HSV challenge.

Protective immunity against HSV challenge

To evaluate the protective efficacy of salbutamol administration against a virulent HSV challenge, groups of challenged mice were examined daily for mortality. Mice that were inoculated with a mixture of pCIgB DNA vaccine and 50 µg salbutamol showed less mortality and significantly prolonged survival against mucosal challenge of virulent virus compared to pCIgB-administered mice (*P* = 0.027) (Figure 7A). The animals immunized with the control vector (pCI-neo) were not protected, whereas mice that were administered with pCIgB DNA vaccine alone had a survival rate of 14.3% 14 days after challenge. Furthermore, mice co-administered

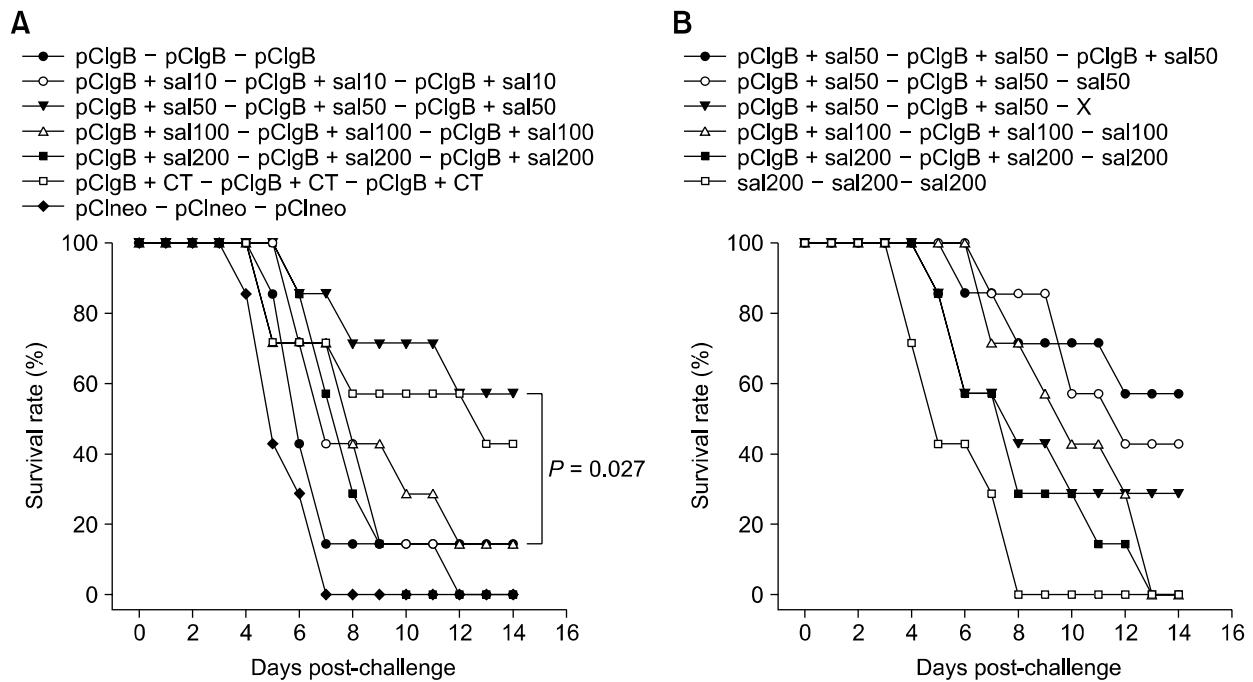
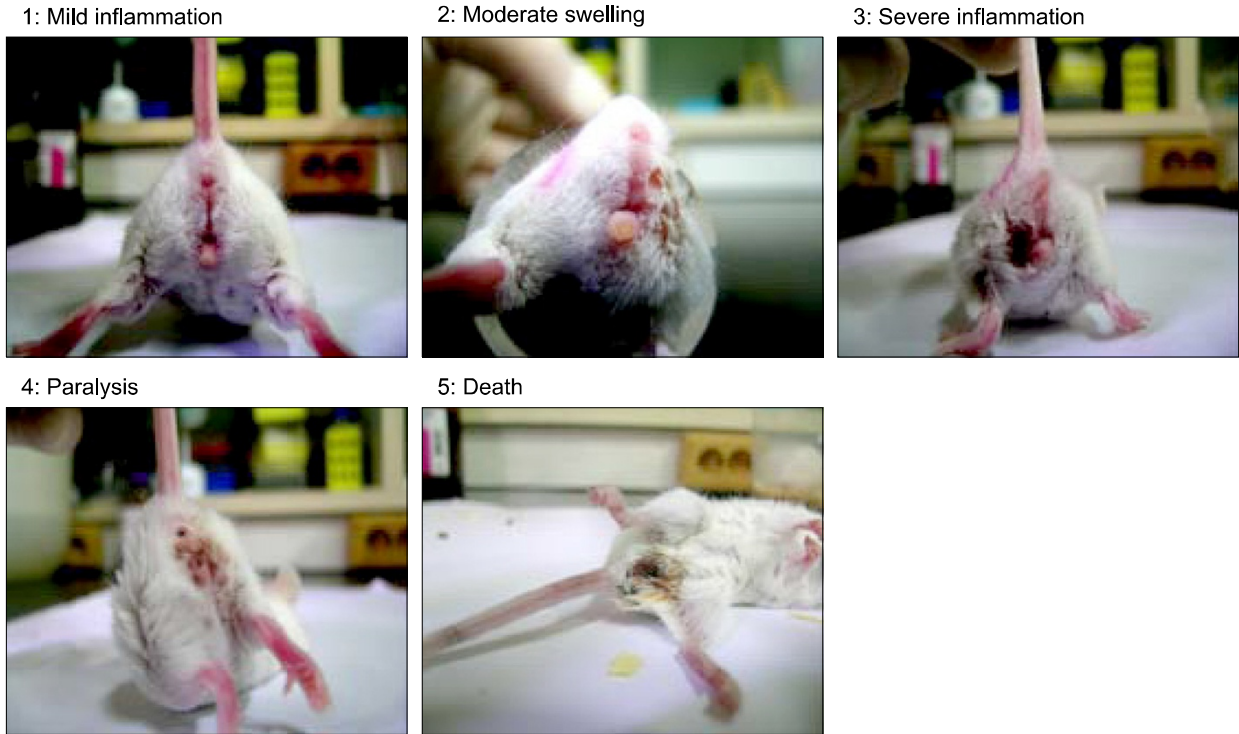


Figure 7. Susceptibility of immunized animals against mucosal challenge of HSV. (A and B) Groups of BALB/c (H-2^d) mice were immunized i.n. with the indicated mixture of pCIgB DNA vaccine (100 µg) and salbutamol (10, 50, 100, and 200 µg) three times at 7-day intervals. After 2-weeks of the final immunization, the mice were challenged intravaginally with HSV McKrae strain (10⁶ PFU). The challenged mice were then examined daily to determine surviving mice until 14 days post-challenge. Kaplan-Meiers survival curves were computed and analyzed using the chi-square test.

A



B

- pClgB - pClgB - pClgB
 - pClgB + sal10 - pClgB + sal10 - pClgB + sal10
 - ▼ pClgB + sal50 - pClgB + sal50 - pClgB + sal50
 - △ pClgB + sal100 - pClgB + sal100 - pClgB + sal100
 - pClgB + sal200 - pClgB + sal200 - pClgB + sal200
 - pClgB + CT - pClgB + CT - pClgB + CT
 - ◆ pClneo - pClneo - pClneo
- pClgB + sal50 - pClgB + sal50 - pClgB + sal50
 - pClgB + sal50 - pClgB + sal50 - sal50
 - ▼ pClgB + sal50 - pClgB + sal50 - X
 - △ pClgB + sal100 - pClgB + sal100 - sal100
 - pClgB + sal200 - pClgB + sal200 - sal200
 - sal200 - sal200 - sal200

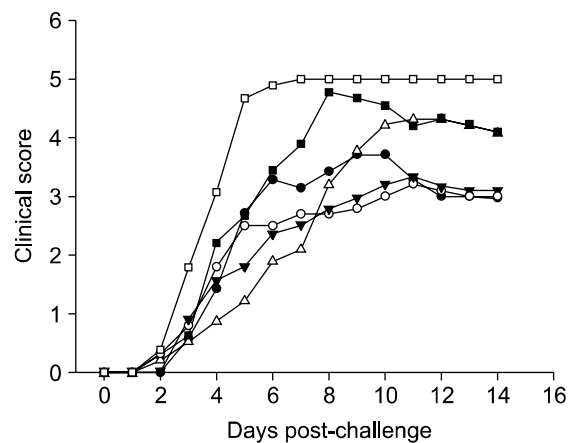
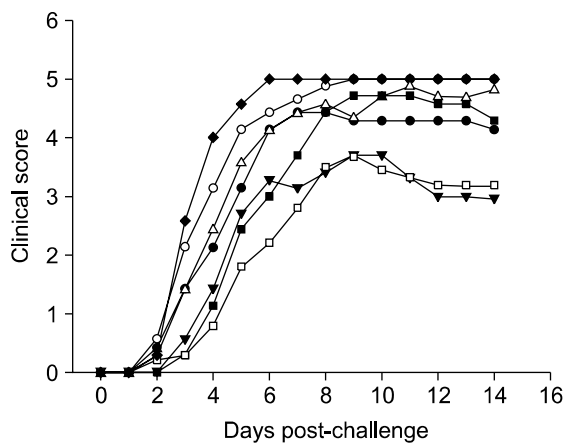


Figure 8. Clinical severity of immunized mice against mucosal challenge of HSV. Groups of BALB/c (H-2^d) mice were immunized i.n. with the indicated mixture of pClgB DNA vaccine (100 μ g) and salbutamol (10, 50, 100, and 200 μ g) three times at 7-day intervals. After 2-weeks of the final immunization, the mice were challenged intravaginally with HSV McKrae strain (10^6 PFU). The challenged mice were then examined daily for vaginal inflammation, neurological illness, and death. (A) Clinical severity was graded as follows: 0, no inflammation; 1, mild inflammation; 2, moderate swelling; 3, severe inflammation; 4, paralysis; 5, death. (B) The average levels of clinical severity. The graph represents the average of clinical scores obtained from seven mice per group.

with 50 µg salbutamol showed the highest survival rate against mucosal challenge of HSV McKrae strain (57.1%). Also, group that received two times of pCIgB plus 50 µg salbutamol mixture showed slightly enhanced protection against HSV challenge compared to pCIgB-administered group (survival rate = 28.5%) (Figure 7B). However, the co-administration of pCIgB DNA vaccine and higher salbutamol doses (100 and 200 µg) could not provide the effective protection against HSV challenge (Figure 7A). Similarly, when the clinical severity of challenged mice was scored according to the clinical sign of inflammation (Figure 8A), the mice that received a mixture of pCIgB DNA vaccine plus 50 µg salbutamol showed significantly reduced inflammation (intensity and duration) (Figure 8B). These results suggest that the i.n. co-administration of salbutamol at optimal doses could provide the effective protection against mucosal challenge of HSV.

Discussion

An aim of mucosal antigen administration is exploitation of common mucosal defense mechanisms and induction of barrier levels of immunity at multiple mucosal surfaces. The most important advantage of mucosal vaccination is the induction of both mucosal and systemic immunity in contrast to parentally delivered vaccine which generally induce poor mucosal immunity (Lamm, 1997). Further, a particular advantage of i.n. vaccine delivery is the requirement of smaller amounts of antigen than oral immunization (Partidos, 2000; Davis, 2001). This is an important concept in the prevention of invasion by pathogens that enter the body via mucosae and damage mucosal sites. However, appropriate mucosal adjuvants for maximal levels of antigen-specific immune responses must be employed in both mucosal and systemic lymphoid tissue compartments, since mucosal delivery generally induces weaker immunity than systemic vaccination (Kuklin *et al.*, 1997; MacLaughlin *et al.*, 1998). Although native CT is an effective mucosal adjuvant in animal models, its innate toxicity limits its use in humans (Freytag and Clements, 2005). In this regard, the β_2 -adrenergic agonist salbutamol may be an ideal candidate adjuvant for mucosal vaccination as described in this study, since salbutamol is currently used as aerosolized anti-asthmatic drugs with a wide safety margin and lack of side effects in their local administration (Hoffman and Lefkowitz, 1990). Here, we demonstrated that salbutamol co-administered with HSV DNA vaccine via i.n. route

enhanced gB-specific antibody responses in both systemic and mucosal tissues, and induced Th₂-biased immune responses against HSV antigen. Moreover, humoral and cellular immune responses modulated by the i.n. co-administration of salbutamol could provide the effective protection and reduced clinical sign against mucosal challenge of HSV. However, the optimal dosages of co-administered salbutamol should be adjusted to induce maximal immune responses in response to HSV. Therefore, our demonstration suggested that β_2 -adrenergic agonists such as salbutamol could offer an attractive possibility for the development of an effective genetic transfer of DNA vaccine via i.n. route.

CT is synthesized as multisubunit toxins with A and B components. The A-subunit is the active moiety which consists of two chains (A1 and A2) joined by a proteolytically sensitive peptide subtended by a disulfide loop. The enzymatic activity of the A1 chain of two chains is highly dependent on a protein co-factor (termed ADP ribosylating factor) which belongs to the regulatory GTPase superfamily. The interaction of A1 and ADP ribosylating factor reduces the binding constants for the A1 substrate NAD⁺ and the α -subunit of one member of the heterotrimeric GTP-binding protein family (G α). G α is incapable of hydrolyzing GTP through normal interaction with GTPase activating protein (GAP) if A1 transfer the ADP-ribose moiety from NAD⁺ to G α (Elson and Ealring, 1984; Lycke, 1993, 1996; Leal-Berumen *et al.*, 1996; Agren *et al.*, 1997). Consequently, adenylyl cyclase as a G α target is irreversibly activated and lead to the elevation of intracellular cAMP. Increased levels of cAMP induce various biochemical changes including activation of PKA. Therefore, it was assumed that β_2 -adrenergic agonist salbutamol, which is widely used as anti-asthmatic drug and induces a temporary cAMP accumulation in target cells (Strosberg, 1997), may be a candidate for mucosal adjuvants. Accordingly, it was likely that salbutamol might induce enhanced immune responses through mechanisms similar to cholera toxin, as proved by cAMP production in DCs. Our demonstration supports the previous finding that showed enhanced immune responses against the major surface protein of *Toxoplasma gondii* when i.n. co-administered with salbutamol (Fermin *et al.*, 1999). However, co-administration of salbutamol (> 50 µg per mouse) unexpectedly decreased immune response maximally induced in response to HSV antigen, which indicating that administered doses must be evaluated to induce optimal immune responses. Conceivably, it is speculated that the adjuvant activity of salbutamol may be caused

by modulating the functions of antigen-presenting cells since β_2 -adrenergic agonists revealed the inhibition of IL-12 secretion in human dendritic cells (Panina-Bordignon *et al.*, 1997; Botta and Maestroni, 2008). This inhibitory effect of IL-12 secretion by β_2 -adrenergic agonist supports our demonstration showing Th2-biased immune responses when co-administered with salbutamol. Moreover, since Th2-biased immune response is beneficial to mucosal vaccination, it is possible that enhanced immune responses in both mucosal and systemic tissues provided effective protection against mucosal challenge of HSV, although CD4⁺ Th1-type responses had an important role in conferring effective protection against HSV infection (Eo *et al.*, 2001a). It was likely that the protective immunity might have been provided by effective and rapid responses to HSV mucosal challenge as indicated by anamnestic responses.

In conclusion, our demonstration suggests that salbutamol displayed adjuvant properties when administered i.n. with DNA vaccines. However, optimization must be performed to induce desired immune responses against HSV antigen. Further, the synergy resulting from combined administration with other adjuvants will result in useful strategies to produce desired outcomes or to redirect such outcomes to more appropriate targets.

Methods

Mice, cells, and viruses

Female 5- to 6-week old BALB/c (H-2d) mice were purchased from KOATECH (Pyeongtaek, Korea) and maintained under standard conditions at the animal facility of Chonbuk National University, according to the Institutional Guidelines. All experiments were conducted according to the guidelines of the committee on the Care of Laboratory Animal Resources, Commission on Life Science, National Research Council. DC2.4, an established dendritic cells (DCs), were kindly provided by Dr. K.L. Rock (Dana-Farber, Boston, MA) and maintained with complete RPMI containing 10% FBS. HSV-1 McKrae strain were propagated in Vero cells (CCL81; ATCC, Manassas, VA) using DMEM supplemented with 2.5% FBS, penicillin (100 U/ml), and streptomycin (100 U/ml). The Vero cells were infected with HSV at a multiplicity of infection (MOI) of 0.01, and then incubated in a humidified CO₂ incubator for 1 h at 37°C. After absorption, the inoculum was removed and 10 ml of a maintenance medium containing 2.5% FBS was added. Approximately 48-72 h after infection, a culture of host cells that showed an 80-90% cytopathic effect was harvested. The virus stocks were then concentrated by centrifugation at 50,000 × *g*, titrated using a plaque assay and stored in aliquots at -80°C until needed.

Plasmid DNA preparation

The plasmid DNA encoding glycoprotein B (gB) of HSV-1 under control of the cytomegalovirus promoter (pCIgB) has been described elsewhere (Eo *et al.*, 2001b). The plasmid DNA used for immunization was purified by polyethylene glycol precipitation using a method that has been described elsewhere (Han *et al.*, 2005; Yoon *et al.*, 2008). Briefly, the cellular proteins were precipitated with 1 volume of 7.5 M ammonium acetate. The supernatant was then precipitated with isopropanol, after which the plasmids were extracted three times with phenol-chloroform and then precipitated with pure ethanol. The DNA quality was then checked by electrophoresis in 1% agarose gel. Next, the concentration of the plasmid DNA was measured using a GeneQuant RNA/DNA calculator (Biochrom, Cambridge, UK). The amount of endotoxin was then determined using the Limulus amoebocyte lysate (LAL) test (< 0.05 EU/ μ g). The *in vivo* effect of endotoxin and the CpG motif was always addressed in parallel by the administration of a control vector.

Immunization and sample collection

Groups of 5- to 6-week old female mice (n = 7) were co-immunized intranasally (i.n.) with 100 μ g of pCIgB in the presence of β_2 -adrenergic agonist, salbutamol (10, 50, 100, and 200 μ g). To examine the effect of plasmid DNA backbone (e.g. CpG motif), some of mice were immunized i.n. with 100 μ g of the control vector (pCI-neo) in parallel. The i.n. immunization was performed three times at 7-day intervals (days 0, 7, and 14) by depositing pCIgB dissolved in a total volume of 20 μ l of PBS (pH 7.2) containing the indicated dose of salbutamol onto the nares of deeply anesthetized mice. Serum samples were collected on the 7th day after each immunization by retro-orbital bleeding and stored at -80°C until needed. Vaginal lavage fluid samples were obtained by introducing 100 μ l of PBS (pH 7.2) into the vaginal canal and recovering it with a micropipette. Vaginal lavages were collected once a day for 3 days, and combined on day 7 post-immunization.

ELISA for gB-specific IgG, IgG1, IgG2a, and IgA

A standard ELISA was used to determine levels of gB-specific antibodies in the serum and vaginal lavage (total IgG, IgG1, IgG2a, and vaginal IgA). Briefly, ELISA plates were coated overnight at 4°C with an optimal dilution (200 ng/well) of purified HSV gB protein in the sample wells, and goat anti-mouse IgG/IgG1/IgG2a (Southern Biotechnology Associate Inc., Birmingham, AL) or rabbit anti-mouse IgA (Zymed, San Francisco, CA) in the standard wells. The plates were washed three times with PBS-Tween 20 (PBST) and blocked with 3% dehydrated milk. The samples were serially diluted twofold, incubated for 2 h at 37°C, and then incubated with HRP-conjugated goat anti-mouse IgG/IgG1/IgG2a for 1 h. To measure the IgA level in the vaginal lavage fluid samples, biotinylated goat-anti-mouse IgA was added for 2 h at 37°C, followed by the addition of peroxidase-conjugated streptavidin (Jackson ImmunoResearch Laboratories, West Grove, PA). The color was developed by the addition of a suitable substrate (11 mg of 2,2-azinobis-3-ethylbenzothia-

zoline-6-sulfonic acid in a mixture of 25 ml of 0.1 M citric acid, 25 ml of 0.1 M sodium phosphate, and 10 μ l of hydrogen peroxide). The concentration of gB-specific antibodies was determined using an automated ELISA reader and the SOFTmax Pro4.3 program (Spectra MAX340, Molecular Device, Sunnyvale, CA).

HSV-specific Th-cell proliferation

HSV-specific Th-cell proliferation was evaluated 14 days after the final immunization. The immunized mice were sacrificed and the splenocytes and draining LN (cervical LNs) cells were prepared. Splenocytes and cervical LN cells were then restimulated with irradiated syngeneic APCs that had been enriched by metrizamide gradient (Eo *et al.*, 2001a) and pulsed with UV-inactivated HSV-1 KOS (1.5 MOI before UV inactivation). Following 3-day incubation, [3 H] thymidine (0.5 mCi/well) was added to the cultures 18 h before harvesting. After freezing and thawing, cells were collected onto glass fibre filters and radioactivity incorporation was measured by liquid scintillation counting. In some experiments, CD4⁺ T cells purified from HSV-immunized mice were used for proliferation. The proliferative responses were tested in quadruplicated well and the results were expressed as mean cpm \pm SD.

cAMP production by DCs treated with β_2 -adrenergic agonist

The intracellular amount of cAMP produced by DCs treated with β_2 -adrenergic agonist, salbutamol, was determined using a direct cAMP enzyme immunoassay kit (Assay Designs, Inc., Ann Arbor, MI). DC2.4 cells (1×10^6) seeded in 6-well culture plates were rinsed with serum-free media during the last 3 h. Subsequently, Hank's balanced salt solution containing 5 mM theophylline was added to block cAMP hydrolysis. After 15 min, 25 μ l of salbutamol serial dilutions were added to the desired final concentrations. The treatment of salbutamol were performed in the presence or absence of β -adrenergic antagonist, D,L-propranolol (10 μ M), for 30 min at 37°C. The treatment was stopped by aspiration of solution and addition of cell lysis solution containing 0.1 M HCl (1 ml). The intracellular cAMP content was determined using direct cAMP assay kit, according to manufacturer's instructions.

Cytokine ELISA

Two weeks after the final immunization, the mice were sacrificed, and the splenocytes and cervical LN cells were prepared. The erythrocytes were depleted by treating the single-cell suspensions with 1.6 M ammonium chloride containing 170 mM Tris-HCl buffer for 5 min at 37°C. These cells were used as the responder cells. The enriched antigen-presenting cell (APC) populations, which were obtained using the method described by Eo *et al.* (2001a), were used as the stimulators. Following treatment of the enriched APCs with mitomycin C (25 μ g/ml), the responder cells and HSV-pulsed APCs were added to 200 μ l of a RPMI medium at responder-to-stimulator ratios of 5:1, 2.5:1, and 1.25:1. The culture supernatants were then harvested after 3-day incubation.

ELISA was used to determine the cytokine levels in the culture supernatants. Briefly, 100 ng/well of either IL-2, IL-4, or IFN- γ anti-mouse antibody (eBioscience, San Diego, CA; clone no. JES6-1A12, 11B11, and R4-6A2, respectively) was added to each ELISA plate. The plates were then incubated overnight at 4°C, after which they were washed three times with PBST and then blocked with 3% nonfat-dried milk for 2 h at 37°C. The culture supernatant and recombinant IL-2, IL-4, and IFN- γ protein (Pharmingen, San Diego, CA) were used as the standards. Each of these reagents was serially diluted two-fold, and then added to the corresponding plates. The plates were then incubated overnight at 4°C. Next, biotinylated IL-2, IL-4, and IFN- γ antibodies (eBioscience; clone no. JES6-5H4, BVD6-24G2, and XMG1.2, respectively) were added, after which the plates were incubated at 37°C for an additional 2 h. The plates were then washed and incubated with peroxidase-conjugated streptavidin (Pharmingen) for 1 h, after which the color was developed by adding a substrate (ABTS) solution. The concentrations of cytokines were then determined using an automated ELISA reader and the SOFTmax Pro4.3 program to compare the samples to two concentrations of standard cytokine protein. Data were expressed by subtracting the produced cytokine levels of no HSV antigen-treated cultures from cytokine produced from HSV-stimulated cultures.

Vaginal challenge

The mice were previously treated with progesterone to synchronize their estrous cycles, as described earlier (Parr *et al.*, 1994). Briefly, BALB/c mice were subcutaneously injected with Depo-Provera (DP) (Upjohn Co., Kalamazoo, MI) at 2 mg per mouse. Five days following the injection of DP, the mice were challenged intravaginally with 10^6 PFU of HSV-1 McKrae. The mice were examined daily for vaginal inflammation, neurological illness, and death, as described previously (Eo *et al.*, 2001a). They were scored 1 to 5 depending on the clinical severity of disease (0, no change; 1, mild inflammation; 2, moderate swelling; 3, severe inflammation; 4, paralysis; 5, death).

Statistical analysis

Where specified, the data were analyzed for statistical significance using Student's *t*-test. A *P* values < 0.05 was considered statistically significant. Also, Kaplan-Meier curves were generated for mice that survived a lethal challenge with HSV-1. The survival time of mice that were alive at the end of the study was regarded as censored. Time data were analyzed using the log rank statistic to compare the two survival curves, and *P* values were computed with the chi-square method.

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