# Ganglioside GM1 Binding Peptides: A Potential Adjuvant for Transcutaneous Immunization

Kristina K. Peachman[*], Danielle M. Mclean, James C. Tong[§], Carl R. Alving and Mangala Rao

*Department of Adjuvant and Antigen Research, Division of Retrovirology, U.S. Military HIV Research Program, Walter Reed Army Institute of Research, Rockville, MD, USA*

**Abstract:** Cholera toxin (CT) binds ganglioside GM1 and has been used as an adjuvant in transcutaneous immunization. To determine if the adjuvant property of CT was merely due to binding to ganglioside GM1, mice were immunized by the transcutaneous route using hen egg lysozyme (HEL) as the antigen, and either CT or synthetic peptides that bind to ganglioside GM1 as the adjuvant, and the immune responses were evaluated. Both CT and GM1 binding peptides (GM1-bp) induced HEL-specific antibodies and T-cell proliferation. However, the immune responses when GM1-bp was used as the adjuvant, was much lower when compared to CT as the adjuvant. GM1-bp maintained or enhanced the co-stimulatory molecules on antigen presenting cells, in particular on JAWS II dendritic cells. Overall, the data suggests that the binding of GM1-bp to ganglioside GM1 alone can induce immune responses to the co-administered antigen.

**Keywords:** Cholera toxin, ganglioside GM1 binding peptides, transcutaneous immunization.

## INTRODUCTION

Transcutaneous immunization (TCI), a needle-free technique, is a method of vaccination by which the antigen along with an adjuvant is applied directly on the skin [1-3]. The antigen and the adjuvant travel through hydrated skin and induce potent cellular, humoral and mucosal immune responses against a wide range of protein and peptide antigens including DNA and influenza antigens [4-10].

The most effective and widely used adjuvants for TCI have been the ADP-ribosylating bacterial exotoxins, including heat-labile enterotoxin (LT) and cholera toxin (CT) [2]. CT and LT are each composed of 5 identical B subunits that bind to the receptor ganglioside GM1 on the cell surface. The B subunits are non-covalently linked to a single A subunit consisting of two domains A1 and A2 [11-12]. While the pathways by which CT and LT act as adjuvants have not been fully elucidated, two attributes of these complex toxins are central to this function, enzymatic ADP-ribosyltransferase activity and high-affinity ganglioside GM1 binding to cell membranes [13]. The A subunit exhibits ADP-ribosyltransferase activity while the pentameric B subunit is responsible for binding the toxins through the receptor. Through ADP ribosylation of G proteins and activation of adenylate cyclase, the enzymatic A1 subunit initiates a cascade of events that promotes an outflow of intracellular fluids (enterotoxicity) and stimulates a number of other less well-understood cell signaling pathways. These signaling pathways potentiate immune responses to co-administered antigens [11]. Dissociation of adjuvant and enterotoxic effects by introduction of mutations in or around the A1 active site have been somewhat successful, although disagreement remains about the contribution of residual enzymatic activity in the adjuvant properties of these mutant toxins [14-16].

The binding of B subunit of CT and LT to GM1 ganglioside is essential to their adjuvant property and is important in directing and properly presenting the molecule to antigen presenting cells [17-19]. Toxin binding to GM1 also triggers events that result in efficient antigen uptake and activation of cell signals that improve antigen presentation [20]. The non-toxic B subunit pentamer of LT or CT has been coupled to antigen as well as co-administered with soluble protein antigen by the intranasal route and shown to induce IgA and IgG-mediated protective mucosal immunity [21-24].

Despite their success as adjuvants in animal studies, the utility of LT and CT in humans has been limited because of their inherent toxicity when administered by the oral or intranasal routes and poses a challenge to their usage [25, 26]. Clinical trials using LT in the transcutaneous immunization platform are ongoing and have to date induced potent immune responses to vaccines and proven to be safe with no toxic side effects [27, 28]. Research to elucidate the molecular basis of adjuvanticity and toxicity with a view to exploit the successful adjuvant properties of the toxin have been undertaken.

Using a phage-displayed pentadecapeptide library, Matsubara *et al.* identified ganglioside GM1 binding peptides (GM1-bp). These small 15mer peptides bound with high affinity to a GM1 monolayer [29]. Remarkably, none of the three peptides displayed any similarity to the GM1 binding site of CTB or LTB, yet all three inhibited the binding of CTB to GM1 monolayers. In addition, Montaner *et al.* [30] described 45 additional GM1-bp. They utilized

*Address correspondence to this author at the Department of Adjuvant and Antigen Research, Division of Retrovirology, 1600 East Gude Drive, Rockville, MD 20850 USA; Phone: (301) 251-5076; Fax: (301) 424-3120; E-mail: kpeachman@hivresearch.org

[§]Current address: Telemedicine & Advanced Technology Research Center, Ft. Detrick, Fredrick, MD, USA

1874-2262/09 2009 Bentham Open

two of the peptides for intranasal immunization with influenza proteins and demonstrated that both mucosal as well as systemic antibody responses were generated [30]. These GM1-bp could prove to be safe and potent adjuvants and may also provide a useful and unique tool to define the specific contribution of GM1 binding to the generation of immune responses.

In this study, we demonstrate that when the antigen HEL along with GM1-bp as the adjuvant is applied on the backs of mice by the transcutaneous route, immune responses are induced to the antigen as well as the adjuvant. However, the responses with GM1-bp were much weaker compared to when CT was used as the adjuvant. These results suggest that binding of ganglioside GM1 alone is sufficient to induce an immune response.

## MATERIALS AND LABORATORY PROCEDURES

### Mice and Reagents

C57BL/6 and BALB/c mice were purchased from Jackson Laboratory (Bar Harbor, ME). Hen egg lysozyme (HEL) was purchased from Sigma-Aldrich (St. Louis, MO). RPMI-1640, fetal bovine serum, L-glutamine, penicillin, streptomycin, and phosphate-buffered saline (PBS) were obtained from Gibco-BRL Life Technologies part of Invitrogen Corp. (Rockville, MD). Three ganglioside GM1 binding peptides (GM1-bp) GA, DP, and VP [29] were commercially purchased from The American Peptide Company. The respective sequences for the three GM1-bps are as follows: GWWYKGRARPVSAVA; DFRRLPGAF WQLRQ; and VWRLLAPPFSNRLLP. Anti-CD80, anti-CD86, anti-H-$2K^b$, anti-H-$2K^d$, anti-$IA^b$, anti-$IA^d$, anti-IL-4 (clone BVD4-11), biotinylated anti-IFNγ (clone XMG 1.2) and biotinylated anti-IL-4 (clone BVD6-24G2) were purchased from BD Biosciences PharMingen (San Diego, CA). Cholera toxin (CT) was purchased from List Biological Laboratories, Inc. (Campbell, CA). Immunopure goat $F(ab')_2$ anti-mouse IgG (H&L) was purchased from Pierce Biotechnology Inc. (Rockford, IL). Ninety six-well U-bottom Immulon-2 polystyrene plates were purchased from Dynatech Laboratories (Chantilly, VA). Phosphatase-labeled goat anti-mouse IgG, substrate p-nitrophenyl phosphate and BCIP/NBT were bought from Kirkegaard & Perry Laboratories (Gaithersburg, MD). Multi-Screen-IP sterile plates were purchased from Millipore (Bedford, MA). Anti-IFNγ (clone RMGG-1) was from BioSource, International, Inc., (Camarillo, CA). Avidin-conjugated alkaline phosphatase was purchased from Vector Laboratories (Burlingham, CA). Murine gamma-interferon was purchased from R&D Systems (Minneapolis, MN).

### Mouse Immunizations

Six to eight-week-old-female BALB/c mice were maintained in a specific-pathogen-free facility. Each group of five mice was housed in an individual cage and given water and food ad libitum. This study was conducted in compliance with the Animal Welfare Act and adhered to the principles enunciated in the Guide for the Care and Use of Laboratory Animals. The investigators used facilities fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International. All animal experimentation was approved by the WRAIR Animal Safety Committee. The backs of the mice were shaved one day prior to immunization with a Wahl razor. The mice were immunized *via* transcutaneous immunization (TCI) [31, 32]. The backs of the mice were hydrated with water before the addition of the antigens in a total volume of 50-110 μL. The groups (Table **1**) included Group 1: naive (no treatment); Group 2: 50 μg CT; Group 3: [100 μg of all three GM1-bp (33.3 μg/peptide)]; Group 4: (100 μg HEL + 50 μg CT); Group 5: [100 μg HEL + 100 μg GM1-bp (33.3 μg/peptide)]; Group 6: [100 μg HEL + 50 μg GM1-bp (16.67 μg/peptide)]. Mice were immunized at weeks 0, 3, and 6. Groups 3, 5, and 6 were boosted again at 9 weeks. Animals were bled at 0, 2, 4, 8, 12, 14 and 16 weeks. Spleens and inguinal lymph nodes were taken at week 16.

**Table 1. BALB/c Mice (5 Mice/Group) were Immunized by the Transcutaneous Route with the Antigen and Adjuvant Shown**

| Group | Antigen | Adjuvant |
|---|---|---|
| 1. Naive | None | None |
| 2. Cholera Toxin (CT) | None | CT (50μg) |
| 3. GM1-bp | None | Total GM1-bp (100μg) (33.3μg/peptide) |
| 4. HEL + CT | HEL (100μg) | CT (50μg) |
| 5. HEL + GM1-bp | HEL (100μg) | Total GM1-bp (100μg) (33.3μg/peptide) |
| 6. HEL + GM1-bp | HEL (100μg) | Total GM1-bp (50μg) (16.67μg/peptide) |

### ELISA

Solid-phase enzyme-linked immunosorbent assays (ELISA) were performed to measure the HEL- and CT-specific IgG antibodies. HEL and CT protein were used as the capture antigens as described previously [33]. Immulon-2 96-well U-bottom polystyrene plate were coated with HEL or CT (2 μg/ml, 0.1 ml/well) in antigen diluent [4 μg/ml boiled casein in phosphate buffered saline (PBS)] overnight at 4-6ºC and then blocked with 0.5% casein in PBS containing 1% Tween 20 (PBS-casein-Tween). Individual mouse serum diluted in PBS-casein-Tween were added to the plates in triplicate wells and incubated overnight at 4-6ºC. After washing with 0.05% Tween 20 in PBS (PBS-Tween), the plates were incubated with phosphatase-labeled goat anti-mouse IgG for 2 h at room temperature and then washed again with PBS-Tween. Substrate (p-nitrophenyl phosphate) was added and the plates were incubated in the dark for 30 min (CT) or 1hr (HEL). Absorbance was read at 405 nm with an Uvmax plate reader (Molecular Devices, Sunnyvale, CA). The data are expressed as endpoint titers defined as the highest dilution that yielded an optical density reading greater than or equal to twice that of the background values.

### Proliferation

Spleen and lymph nodes obtained from naive and immunized mice were cultured in complete RPMI 1640 media containing 0.5% normal mouse serum. Cultures were set up in 96-well plates in triplicate in 0.2 ml volumes at a concentration of $5 \times 10^5$ cells/ml for spleen cells or at a

concentration of 2.5 x $10^5$ cells/ml for lymph node cells in the presence or absence of HEL or GM1-bp (1, 3, 10, and 30 μg/ml, respectively) for 5 days. During the last 16 h of the culture period, cells were pulsed with 1 μCi of $^3$[H]thymidine per well [34]. Cells were then harvested onto glass fiber filters. The data are expressed as stimulation indices [$^3$(H)thymidine incorporation in the presence of antigen divided by the same in the absence of antigen] plus or minus standard error.

### ELISPOT

Spleen and lymph node cells secreting IFN-γ and IL-4 were analyzed by ELISPOT as previously described [34]. Briefly, single cell suspensions were prepared from the spleen and lymph nodes of naive and immunized mice (3 mice/group). Spleen cells (1 x $10^6$ cells/well) and lymph node cells (2.5 x $10^5$ cells/well) were plated on anti-IFNγ and anti-IL-4 coated 96-well nitrocellulose-backed MultiScreen-IP sterile plates. Cells were incubated with or without HEL (30μg/ml) for 18 h at 37ºC in a humidified $CO_2$ incubator. Plates were washed, overlaid with 0.125 μg/ml of either biotinylated anti-IFN-γ or biotinylated anti-IL-4, and incubated at RT for 2 h. The plates were then washed and incubated with 1:1000 dilution of avidin-conjugated alkaline phosphatase for 2 h at RT. The plates were washed and bound IFNγ or IL-4 was detected by the addition of BCIP/NBT. The plates were washed with water and the individual spots were visualized and counted the next day using a stereo-binocular microscope. The average number of spots/number of cells plated was calculated.

### Preparation of Antigen Presenting Cells (APCs)

Bone marrow-derived macrophages (BMs) were cultured from the marrow of C57BL/6 mice (H-$2^b$) or BALB/c mice (H-$2^d$). Cells were grown on either acid-washed circular glass coverslips or in 100 mm tissue culture plates as previously described [35]. On day 9, macrophages were supplemented with 10 U/ml of murine interferon gamma (IFN-γ) and used as APCs the next day.

### Surface Marker Expression

On day 9, primary bone marrow derived-murine macrophages and JAWS II dendritic cells were treated with or without IFN-γ (10 units/ml) and with or without 1 μg/ml of CT or 1 μg/ml of GM1-bp (0.333 μg/ml per peptide). Cells were incubated overnight at 37ºC. The following day, cells were scrapped and placed in 96-well U-bottom plates (~500,000 cells /well). Cells were washed in PBS containing $Ca^{2+}$ and $Mg^{2+}$. Non-specific binding was blocked with 1:100 goat serum in PBS for 5 min on ice. Cells were then incubated with 4 μg/ml anti-H-$2K^d$, anti-$IA^d$ for BALB/c mice or anti-H-$2K^b$ and anti-$IA^b$ for C57BL/6 mice or anti-CD80 (B7-1) or anti-CD86 (B7-2) for 1 h at 4ºC. Cells were washed, followed by the addition of 4 μg/well of purified FITC-conjugated goat F(ab')$_2$ anti-mouse IgG. Cells were incubated at 4ºC for 1 h, washed in PBS and then analyzed on a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA). The results were analyzed using CellQuest (Becton Dickinson, San Jose, CA) software.

## RESULTS

### HEL and CT Specific Antibody Responses

Mice were immunized by the transcutaneous route with HEL as the antigen and either CT or GM1-bp as the adjuvant. Individual serum samples were analyzed for the presence of HEL and CT-specific antibodies at weeks 4, 8, 12, and 16 by ELISA.

As shown in Fig. (**1A**), mice immunized with HEL and CT (Group 4) showed consistently high HEL-specific IgG titers from weeks 4 through 16 with an endpoint titer of 153,600 at week 16. HEL-specific IgG antibodies were also induced in mice immunized with GM1-bp (Groups 5 and 6) as the adjuvant (Fig. **1B**). However, the titers were significantly lower in Group 5 ($p \leq 0.04$) and the response was delayed by 4 weeks compared to Group 4. Only one out of 5 mice induced HEL-specific IgG antibodies when immunized with the lower dose of GM1-bp (Fig. **1B**, open bars), whereas four out of the five mice induced HEL-specific IgG antibodies when immunized with the higher dose of GM1-bp

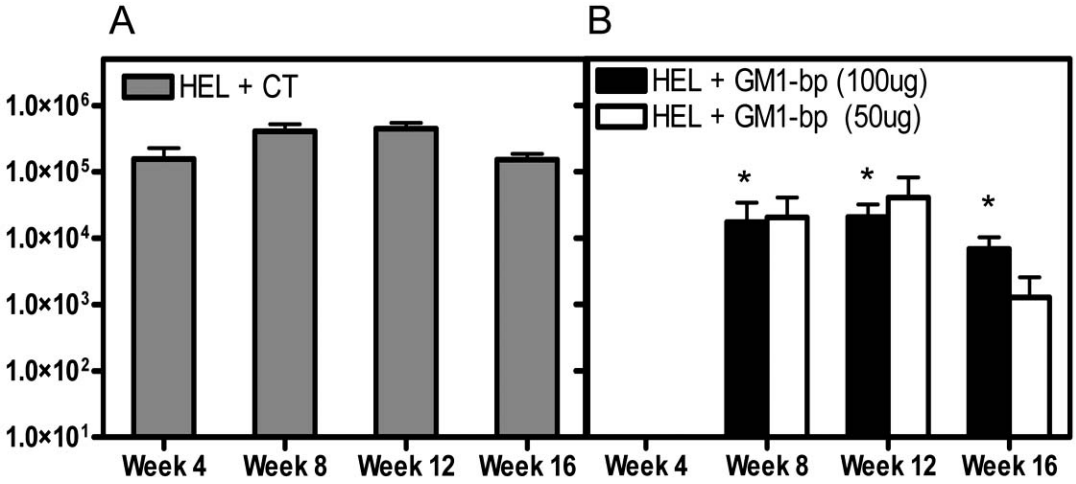


**Fig. (1).** GM1-bp serve as an adjuvant for the induction of HEL-specific IgG antibodies. (**A**) HEL-specific IgG ELISA endpoint titers (gray bars) for Group 4 (100 μg HEL + 50 μg CT) at weeks 4, 8, 12 and 16 post-immunization. (**B**) HEL-specific IgG ELISA endpoint titers for Group 5 (100 μg HEL + 100 μg GM1-bp) [black bars] and Group 6 (100 μg HEL + 50 μg GM1-bp) [white bars]. Each bar represents the mean end point titers of 5 mice ± SEM of each group. *represents statistically significant difference compared to Group 4 ($p \leq 0.04$).

either by coupling or conjugating the peptides to strengthen its adjuvant activity in order to enhance the immune responses to the administered antigen.

## ACKNOWLEDGEMENTS

We gratefully acknowledge the assistance of Ms. Elaine Morrison for performing all of the animal immunization and sera collection.

## DISCLAIMERS

The views expressed in this article are those of the authors and do not reflect the official policy of the Department of the Army, the Department of Defense, or the U.S. government.

## REFERENCES

[1] Glenn GM, Rao M, Matyas GR, Alving CR. Skin immunization made possible by cholera toxin. Nature 1998; 391: 851.

[2] Scharton-Kersten T, Glenn GM, Vassel R, Yu J, Walwender D, Alving CR. Principles of transcutaneous immunization using cholera toxin as an adjuvant. Vaccine 1999; 17(2): S37.

[3] Hammond S, Guebre-Xabier M, Yu J, Glenn GM. Transcutaneous immunization: an emerging route of immunization and potent immunostimulation strategy. Crit Rev Ther Drug Carrier Sys 2001; 18(5): 503-26.

[4] Pye D, Vandenberg KL, Dyer SL, *et al.* Selection of an adjuvant for vaccination with the malaria antigen MSA-2. Vaccine 1997; 15(9): 1017-23.

[5] Rao M, Matyas GR, Vancott TC, Birx DL, Alving CR. Immunostimulatory CpG motifs induce CTL responses to HIV type I oligomeric gp140 envelope protein. Immunol Cell Biol 2004; 82(5): 523-30.

[6] Richards RL, Rao M, Vancott TC, Matyas GR, Birx DL, Alving CR. Liposome-stabilized oil-in-water emulsions as adjuvants: increased emulsion stability promotes induction of cytotoxic T lymphocytes against an HIV envelope antigen. Immunol Cell Biol 2004; 82(5): 531-8.

[7] Matyas GR, Friedlander AM, Glenn GM, Little S, Yu J, Alving, CR. Needle-free skin patch vaccination method for anthrax. Infect Immun 2004; 72(2): 1181-83.

[8] Peachman KK, Rao M, Alving CR, *et al.* Correlation between lethal toxin-neutralizing antibody titers and protection from intranasal challenge with Bacillus anthracis Ames strain spores in mice after transcutaneous immunization with recombinant anthrax protective antigen. Infect Immun 2006; 74(1): 794-7.

[9] Vogt A, Mahé B, Costagliola D, *et al.* Transcutaneous anti-influenza vaccination promotes both CD4 and CD8 T cell immune responses in humans. J Immunol 2008; 180(3): 1482-9.

[10] Mkrtichyan M, Ghochikyan A, Movsesyan N, *et al.* Immunostimulant adjuvant patch enhances humoral and cellular immune responses to DNA immunization. DNA Cell Biol 2008; 27(1): 19-24.

[11] Spangler BD. Structure and function of cholera toxin and the related *Escherichia coli* heat-labile enterotoxin. Microbiol Rev 1992; 56(4): 622-47.

[12] Zhang RG, Scott DL, Westbrook ML, *et al.* The three-dimensional crystal structure of cholera toxin. J Mol Biol 1995; 251(4): 563-73.

[13] Freytag LC, Clements JD. Bacterial toxins as mucosal adjuvants. Curr Top Microbiol Immunol 1999; 236: 215-36.

[14] Dickinson BL, Clements JD. Dissociation of *Escherichia coli* heat-labile enterotoxin adjuvanticity from ADP ribosyltransferase activity. Infect Immun 1995; 63(5): 1617-23.

[15] Cheng E, Cardenas-Freytag L, Clements JD. The role of cAMP in mucosal adjuvanticity of *Escherichia coli* heat-labile enterotoxin (LT). Vaccine 1999; 18(1-2): 38-49.

[16] Pizza M, Giuliani MM, Fontana MR, *et al.* Mucosal vaccines: non toxic derivatives of LT and CT as mucosal adjuvants. Vaccine 2001; 19(17-19): 2534-41.

[17] Nashar TO, Betteridge ZE, Mitchell RN. Evidence for a role of ganglioside GM1 in antigen presentation: binding enhances presentation of *Escherichia coli* enterotoxin B subunit (EtxB) to CD4(+) T cells. Int Immunol 2001; 13(4): 541-51.

[18] Holmgren J, Czerkinsky C, Lycke N, Svennerholm AM. Strategies for the induction of immune responses at mucosal surfaces making use of cholera toxin B subunit as immunogen, carrier, and adjuvant. Am J Trop Med Hyg 1994; 50: 42-54.

[19] Guidry JJ, Cardenas L, Cheng E, Clements JD. Role of receptor binding in toxicity, immunogenicity, and adjuvanticity of *Escherichia coli* heat-labile enterotoxin. Infect Immun 1997; 65(12): 4943-50.

[20] de Haan L, Hirst TR. Cholera toxin: a paradigm for multi-functional engagement of cellular mechanisms. Mol Membr Biol 2004; 21(2): 77-92.

[21] Richards CM, Aman AT, Hirst TR, Hill TJ, Williams NA. Protective mucosal immunity to ocular herpes simplex virus type 1 infection in mice by using *Escherichia coli* heat-labile enterotoxin B subunit as an adjuvant. J Virol 2001; 75(4): 1664-71.

[22] de Haan L, Verweij WR, Holtrop M, *et al.* Nasal or intramuscular immunization of mice with influenza subunit antigen and the B subunit of *Escherichia coli* heat-labile toxin induces IgA- or IgG-mediated protective mucosal immunity. Vaccine 2001; 19(20-22): 2898-907.

[23] Rask C, Fredriksson M, Lindblad M, Czerkinsky C, Holmgren J. Mucosal and systemic antibody responses after peroral or intranasal immunization: effects of conjugation to enterotoxin B subunits and/or of co-administration with free toxin as adjuvant. APMIS 2000; 108(3): 178-86.

[24] George-Chandy A, Eriksson K, Lebens M, Nordstrom I, Schon E, Holmgren J. Cholera toxin B subunit as a carrier molecule promotes antigen presentation and increases CD40 and CD86 expression on antigen-presenting cells. Infect Immun 2001; 69(9): 5716-25.

[25] Frech SA, Dupont HL, Bourgeois AL, *et al.* Use of a patch containing heat-labile toxin from *Escherichia coli* against travellers' diarrhoea: a phase II, randomised, double-blind, placebo-controlled field trial. Lancet 2008; 371(9629): 2019-25.

[26] McKenzie R, Bourgeois AL, Frech SA, *et al.* Transcutaneous immunization with the heat-labile toxin (LT) of enterotoxigenic *Escherichia coli* (ETEC): protective efficacy in a double-blind, placebo-controlled challenge study. Vaccine 2007; 25(18): 3684-91.

[27] Fujihashi K, Koga T, van Ginkel FW, Hagiwara Y, McGhee JR. A dilemma for mucosal vaccination: efficacy versus toxicity using enterotoxin-based adjuvants. Vaccine 2002; 20(19-20): 2431-8.

[28] Mutsch M, Zhou W, Rhodes P, *et al.* Use of the inactivated intranasal influenza vaccine and the risk of Bell's palsy in Switzerland. N Engl J Med 2004; 350(9): 896-903.

[29] Matsubara T, Ishikawa D, Taki T, Okahata Y, Sato T. Selection of ganglioside GM1-binding peptides by using a phage library. FEBS Lett 1999; 465(2): 253-56.

[30] Montaner AD, De Nichilo A, Elias F, *et al.* Ganglioside GM1-binding peptides as adjuvants of antigens inoculated by the intranasal route. Vaccine 2006; 24(11): 1889-96.

[31] Scharton-Kersten T, Yu J, Vassell R, O'Hagan D, Alving CR, Glenn GM. Transcutaneous immunization with bacterial ADP-ribosylating exotoxins, subuits, and unrelated adjuvants. Infect Immun 2000; 68(9): 5306-13.

[32] Glenn GM, Scharton-Kersten T, Vassell R, Matyas GR, Alving CR. Transcutaneous immunization with bacterial ADP-ribosylating exotoxins as antigens and adjuvants. Infect Immun 1999; 67(3): 1100-6.

[33] Rao M, Matyas GR, Vancott TC, Birx DL, Alving CR. Immuno-stimulatory CpG motifs induce CTL responses to HIV type I oligomeric gp140 envelope protein. Immunol Cell Biol 2004; 82(5): 523-30.

[34] Rao M, Bray M, Alving CR, Jahrling P, Matyas GR. Induction of immune responses in mice and monkeys to Ebola virus after immunization with liposome-encapsulated irradiated Ebola virus: protection in mice requires CD4(+) T cells. J Virol 2002; 76(18): 9176-85.

[35] Rao M, Rothwell SW, Wassef NM, Pagano RE, Alving CR. Visualization of peptides derived from lipsome-encapsulated proteins in the trans-Golgi area of macrophages. Immunol Lett 1997; 59(2): 99-105.

[36] Gattoni A, Parlato A, Vangieri B, Bresciani M, Derna R. Interferon-gamma: biologic functions and HCV therapy (type I/II) (1 of 2 parts). Clin Ter 2006; 157(4): 377-86.

[37] Ganguly NK, Kaur T. Mechanism of action of cholera toxin & other toxins. Indian J Med Res 1996; 104: 28-37.
[38] Couch RB. Nasal vaccination, *Escherichia coli* enterotoxin, and Bell's palsy. N Engl J Med 2004; 350(9):860-1.
[39] Frech SA, Kenney RT, Spyr CA, *et al.* Improved immune responses to influenza vaccination in the elderly using an immunostimulant patch. Vaccine 2005; 23(7): 946-50.
[40] Spangler BD. Structure and function of cholera toxin and the related *Escherichia coli* heat-labile enterotoxin. Microbiol Rev 1992; 56(4): 622-47.
[41] Cong Y, Weaver CT, Elson CO. The mucosal adjuvanticity of Cholera toxin involves enhancement of costimulatory activity by selective up-regulation of B7.2 Expression. J Immunol 1997; 159(11): 5301-08.
[42] de Haan L, Feil IK, Verweij WR, Agsteribbe E, Wilschut J. Mutational analysis of the role of ADP-ribosylation activity and GM1-binding activity in the adjuvant properties of the *Escherichia coli* heat-labile enterotoxin towards intranasally administered keyhole limpet hemocyanin. Eur J Immunol 1998; 28(4): 1243-50.
[43] Aman AT, Fraser S, Merritt EA, *et al.* A mutant cholera toxin B subunit that binds GM1- ganglioside but lacks immunomodulatory or toxic activity. Proc Natl Acad Sci USA 2001; 98(15): 8536-41.
[44] Anjuère F, Luci C, Lebens M, *et al. In vivo* adjuvant-induced mobilization and maturation of gut dendritic cells after oral administration of cholera toxin. J Immunol 2004; 173(8): 5103-11.
[45] Fraser SA, de Haan L, Hearn AR, *et al.* Mutant *Escherichia coli* heat-labile toxin B subunit that separates toxoid-mediated signaling and immunomodulatory action from trafficking and delivery functions. Infect Immun 2003; 71(3): 1527-37.

Received: May 29, 2009 Revised: August 6, 2009 Accepted: August 13, 2009