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Alpha-Galactosylceramide is an effective mucosal adjuvant for repeated intranasal or oral delivery of HIV peptide antigens

Amy N. Courtney^A, Pramod N. Nehete^B, Bharti P. Nehete^B, Prakash Thapa^C, Dapeng Zhou^C, and K. Jagannadha Sastry^{A,C}

^ADepartment of Immunology, Unit 901, The University of Texas M.D. Anderson Cancer Center, Houston, TX 77030, USA

^BDepartment of Veterinary Sciences, The University of Texas M.D. Anderson Cancer Center, Bastrop, TX 78602, USA

^CDepartment of Melanoma Medical Oncology, Unit 904, The University of Texas M.D. Anderson Cancer Center, Houston, TX 77030, USA

Abstract

Mucosal delivery of vaccines against sexually transmitted pathogens is important to elicit strong immune responses at biologically relevant sites. However, inclusion of appropriate adjuvants is essential to overcome the inherent mucosal tolerance. We present evidence in support of the effectiveness of co-administering alpha-Galactosylceramide (α -GalCer) as an adjuvant with a CTL-inducing HIV envelope peptide, via either oral or intranasal route, to prime antigen-specific immune responses in multiple systemic and mucosal compartments. Contrary to the known potential of repeated parenteral dosing with α -GalCer to induce NKT cell anergy that could compromise adoptive immunity development, we have observed that two and three doses delivered by the intranasal or oral route were more efficient in priming broader antigen-specific immune responses. These results demonstrate the effectiveness of α -GalCer as adjuvant for repeated intranasal or oral administration of vaccines for protection against mucosally transmitted pathogens.

Keywords

Mucosal Immunization; α -Galactosylceramide; Cellular Immunity

1. INTRODUCTION

The development of a vaccine against Human Immunodeficiency Virus (HIV) is imperative to stop the world-wide AIDS epidemic. It has been estimated that as much as 90% of HIV infections occur by sexual transmission through genital mucosal surfaces [1]. Additionally, it has been suggested that the gut is the primary target of HIV infection, even in cases where infection is by the intravenous (iv) route [1, 2]. Therefore the development of an HIV vaccine should focus on eliciting a strong immune response at the mucosal surfaces,

particularly the gut and gut associated lymph nodes, in addition to the systemic response. Recent studies have shown that mucosal immunization is effective in eliciting strong antigen-specific immune responses at mucosal sites that are less accessible but more biologically relevant, such as the genital mucosal tissues [3].

For mucosal immunization, particularly by the oral route, to be successful it is important to overcome the inherent tolerance. Weakly immunogenic antigens delivered by the mucosal route tend to induce tolerance instead of a robust immune response [3]. Therefore, it is necessary to include adjuvants that, when administered by the mucosal route, can increase the immunogenicity of co-administered antigens. One important aspect of any adjuvant is its capacity to activate the innate immune response, which in turn aids the development of a strong adoptive immunity. Typical mucosal adjuvants include bacterial toxins such as cholera toxin (CT) and the heat-labile enterotoxin from *Escherichia coli* (LT), bacterial unmethylated-CpG DNA, and virus-like particles (VLPs) [4]. These adjuvants typically stimulate the innate immune response through activating dendritic cells (DC), the professional antigen presenting cells (APC). For example, CpG DNA interacts with toll-like receptors (TLR) located on DC and CT has been shown to induce DC migration within Peyer's Patches [4, 5].

The glycolipid α -Galactosylceramide (α -GalCer) is a potent inducer of Natural Killer T (NKT) cells, an important innate immune cell type. This synthetic glycolipid is presented by the CD1d molecules on the APC to NKT cells, which leads to activation and expansion of NKT cells. α -GalCer activated NKT cells have been shown to induce full maturation of DC in the spleen after i.v. immunization. Recently it has been shown that α -GalCer acts as an adjuvant to induce T-cell immunity to both cancer cells and viruses [6, 7] through efficient activation/maturation of the DC. However, studies have shown that when administered by iv or ip immunization, a single dose of α -GalCer can induce hyporesponsive NKT cells which are unresponsive to additional doses of α -GalCer [8, 9]. An alternate strategy to avoid the NKT cell anergy includes delivering *ex vivo* α -GalCer-loaded DC [8]. Therefore, it has been suggested that the presentation of α -GalCer on an alternative APC may lead to the NKT anergy [8]. In fact, B-cells loaded with α -GalCer and injected i.v. were shown to cause NKT anergy [9].

In this study, we present evidence showing that α -GalCer can serve as an efficient mucosal adjuvant for peptide antigens to induce mucosal and systemic cell-mediated immunity after immunization by intranasal or oral route. Furthermore, we observed that repeated dosing with α -GalCer via mucosal routes is beneficial to the induction of broad antigen-specific systemic and mucosal immune responses.

2. MATERIALS AND METHODS

2.1. Animals

Female Balb/C mice aged 6-8 weeks age were purchased from NCI. All procedures on the animals were carried out in accordance with institutionally approved protocols. The animals were housed in micro isolator cages and provided with sterile food and water. The animal facility is fully accredited by the Association for Assessment and Accreditation of

Laboratory Animals Care International. The studies were conducted according to National Institute of Health Guidelines on the care and use of Laboratory Animals.

2.2. Cell Lines and cell cultures

The cell line P815 (DBA/2, H-2^d, Mastocytoma) was maintained in RPMI complete media (CM) supplemented with 10% heat inactivated FBS, 50 U/ml of penicillin-streptomycin and 50µg/ml gentamycin.

2.3. Peptides

Synthetic peptides corresponding to known CTL epitopes from the V3 region of the HIV envelope protein gp120 from HIV-1 IIIB were used in the study for priming immune responses in association with α -GalCer for mucosal immunization of mice. The immunizing peptide was R15K, RIQRGPGRAFTIGK [10] and the specific H-2^d epitope peptide R10I within the R15K sequence was RGPGRFAFVTI [11]. All the peptides were synthesized by the solid-phase method of Merrifield [12] either on a modified Vega250 automatic peptide synthesizer (Vega Biochemicals, Tucson, Arizona) or by the 'Bag' method as described by Houghten [13]. The peptides were >90% pure as determined by high-pressure liquid chromatography (HPLC) and mass spectrometry analyses.

2.4. α -Galactosylceramide (α -GalCer)

α -GalCer was purchased from Alexis Biochemicals (San Diego, CA) and dissolved in dimethyl sulfoxide, DMSO (Sigma).

2.5. Immunizations

Mice were immunized by the intranasal or oral route 1-3 times at 5-day intervals, as reported earlier [14], with a combination of the CTL inducing R15K peptide [10] at 100 µg/mouse/dose and the synthetic glycolipid α -GalCer at 2 µg/mouse/dose. For intranasal immunizations, mice were anaesthetized by the intraperitoneal (ip) injection of ketamine-xylazine mixture, and 10 µl of the adjuvant-antigen mixture in PBS was introduced into each nostril. For oral immunizations, mice were anaesthetized ip with ketamine-xylazine mixture and 100µl of mixture was introduced directly to the mouth. Five days after the last immunization mice were sacrificed and cell suspensions were prepared from the spleen and lymph nodes by homogenization or enzymatic dissociation using collagenase type IV (Sigma).

2.6 Analyses of antigen-specific cytolytic activity by the ⁵¹Cr Release Assay

The cytotoxic T lymphocyte (CTL) responses in single cell suspension from spleens of immunized mice were assayed as described previously [10]. Briefly, spleen cells were re-stimulated for 5 days with R15K or R10I. These effector cells were tested for cytolytic activity against ⁵¹Cr-labeled syngeneic P815 target cells that were pre-incubated with either medium alone, R15K, or R10I. The percentage (%) of specific lysis was calculated using the following formula: % specific lysis = (experimental release - spontaneous release)/ (maximum release-spontaneous release) × 100, where the spontaneous release represents the radioactivity obtained when the target cells were incubated in culture medium without

effectors and maximum release represents the radioactivity obtained when the target cells were lysed with 1 % Triton X-100.

2.7. Preparation of target cells expressing HIV env gp160

The P815 target cells were infected with control (vSC8) or recombinant vaccinia virus (vPE16) expressing the envelope gene from HIV-1IIIB, at a multiplicity of infection of 10 as described earlier [15]. After overnight infection, the cells were washed and labeled with ^{51}Cr and used as targets in the CTL assay. The control and recombinant vaccinia viruses were obtained through the AIDS Research and Reference Reagent Program, division of AIDS, NIAID, NIH.

2.8. IFN- γ ELISpot Assay

Cells isolated from spleen, cervical lymph nodes (CLN), mesenteric lymph nodes (MLN), lung and mediastinal lymph nodes (MdLN) of immunized mice were subjected to ELISpot assay for antigen specific IFN- γ -producing cells as described earlier [16] using the reagent kit from BD Biosciences (San Jose, CA). The spots, representing individual interferon gamma (IFN- γ)-producing cell as spot forming cells (SFC), on the membrane were enumerated by Zellnet Consulting Inc., New York, NY using the KS-ELISPOT automatic system (Carl Zeiss Inc., Thornwood, NY). Responses were considered positive when they were above 10 SFC/well and at least double the number obtained in cells cultured with medium alone.

3. RESULTS AND DISCUSSION

3.1. Intranasal Immunization with a single dose of α -GalCer and the HIV peptide elicits antigen-specific immune responses

α -GalCer was tested as an adjuvant for intranasal immunization of Balb/C mice with R15K, a CTL-inducing HIV-1 envelope peptide [10, 14]. Because repeated parenteral dosing (iv or ip) of α -GalCer causes anergy of the NKT cells [8, 9], the main innate immune target cell type, we tested an immunization regimen consisting of delivering a single dose of α -GalCer ad-mixed with the R15K peptide and harvesting tissues after a 5 days. This protocol resulted in moderate induction of CTL activity that is specific to the immunizing peptide R15K as well as the H-2^d epitope R10I within the R15K sequence, in the spleen (Fig. 1B). However, significant peptide-specific IFN- γ producing cells were not detected in the spleen or gut associated mesenteric lymph nodes (MLN) as shown in Fig. 1C, and only low levels of IFN- γ producing cells were observed in the lung draining mediastinal lymph nodes, MdLN (data not shown). These data, showing weak immunogenicity of the single dose regimen of the HIV model peptide and α -GalCer delivered by the intranasal route, prompted testing whether additional doses of the antigen-adjuvant mixtures would be more immunogenic.

3.2. Repeated intranasal immunization with α -GalCer and the HIV peptide elicits strong peptide-specific systemic and mucosal immune responses

An immunization regimen consisting of delivering α -GalCer ad-mixed with the R15K peptide two times at 5 day interval resulted in strong induction of antigen-specific CTL activity in the spleen (Fig. 2B). Furthermore, we observed significant levels of antigen-

specific IFN- γ producing cells within the mucosal tissues such as the mesenteric lymph nodes (MLN) as shown in Fig. 2C and in multiple other draining lymph nodes such as the cervical lymph node (CLN) and MdLN (data not shown). These data demonstrate that intranasal immunization with α -GalCer ad-mixed with the R15K peptide was effective in priming *not only* a strong systemic immune response, but also a broader mucosal immune response in multiple tissues, including the more distal mucosal tissues such as the MLN. These results suggest that a second intranasal immunization with α -GalCer and R15K is beneficial to induce broader peptide-specific mucosal immunity. Based on these data, we tested whether delivering a third dose of adjuvant-antigen mixture by the intranasal route would further improve systemic and mucosal peptide-specific immune responses. Mice were immunized with α -GalCer and R15K by the intranasal route three times at a 5-day interval and the cells collected from spleen and the various mucosal tissues were analyzed for immune responses. As shown in Fig. 2D, strong peptide-specific CTL response, at comparable levels to that seen after the 2-dose immunization scheme were observed in the spleens. Additionally, peptide-specific IFN- γ producing cells were observed in the spleen as well as MLN (Fig. 2E). These results suggest that repeated dosing with the antigen-adjuvant mixture by the intranasal route is efficient in inducing strong antigen-specific systemic as well as cellular immune responses. In general, there is no significant improvement or hindrance to the CTL responses with the third dose, except at the 100:1 E:T ratio, where the values reached statistical significance ($P < 0.05$). Induction of peptide-specific immune responses in the gut-associated tissues is especially important because in primary HIV infection severe depletion of CD4⁺ T-cells occurs in the gut associated lymphoid tissues (GALT), while Long-Term Non-Progressors (LTNP) maintain normal levels of CD4⁺ T-cells in the GALT which correlate with undetectable viral loads and stable CD4⁺ T-cells in the blood [17]. Similar results showing severe CD4⁺ T-cell depletion in the gut of SIV infected Rhesus Macaques as well as Sooty Mangabeys were reported [18, 19]. These studies suggest that, during primary infection, a majority of the targets for viral infection, replication and persistence are located in the intestinal mucosal tissues and immunization strategies should target these tissues to stimulate effector cells that would eliminate viral persistence [20].

3.3. Repeated oral immunization with α -GalCer and the model HIV peptide results in strong peptide-specific systemic and mucosal immune responses

To determine whether α -GalCer would be an effective adjuvant for oral immunization, the R15K peptide was co-administered by delivering directly into the mouths of Balb/C mice using a pipette two times at a 5-day interval. Antigen-specific CTL response was observed in the spleens of the mice (Fig. 3B). However, no IFN- γ producing cells were detected in all tissues tested including MLN (Fig. 3C). These results were similar to those observed in mice after a single intranasal immunization described above. Therefore, we tested an oral immunization regimen delivering the mixture of α -GalCer and R15K three times at a 5-day interval and observed a similar CTL response within the spleen with no statistical significance to that seen after two dose-immunization regimen (Fig. 3D). However, we observed antigen-specific IFN- γ producing cells specifically in the spleen and the MLN after the third dose of oral immunization (Fig. 3E). These results suggest that a three dose immunization scheme is beneficial to induce efficient and *broader* systemic and mucosal antigen-specific immune responses after oral delivery of α -GalCer and R15K.

3.4. Immunization with the HIV peptide and α -GalCer by either the oral or intranasal route leads to immune recognition of the cognate HIV envelope protein

Splenocytes from mice immunized with α -GalCer and R15K were also tested for their capacity to lyse target cells that had been infected with a recombinant vaccinia virus expressing the HIV-1 envelop protein gp160 (vPE16). Compared to cells infected with the control vaccinia virus vSC8, higher levels of lysis of vPE-infected target cells were observed (Fig. 4B). Mice immunized by either the intranasal or oral routes were both able to elicit envelope-specific lysis and there was no significant difference between the two immunization routes at the higher E:T ratios. These results demonstrate the effectiveness of the mucosal immunization regimen employing α -GalCer adjuvant for R15K peptide to prime cognate antigen-specific CTL responses.

3.5. Repeated Dosing of α -GalCer via mucosal routes does not adversely affect the peptide-specific CTL responses

It has been shown that repeated iv or ip dosing with free α -GalCer leads to NKT cell anergy [8, 9] which may be harmful to the induction of a strong antigen-specific adoptive immune response. To examine how boosting without α -GalCer would effect peptide-specific immune responses, splenocytes from mice immunized with either 2 doses of α -GalCer and R15K or 1 dose of the antigen-adjuvant mixture and a booster 5 days later with the antigen R15K alone were assayed for CTL activity. As shown in Fig. 5, in the case of mice immunized by either the oral or intranasal route, the second dose of α -GalCer did not hamper the induction of peptide-specific immune responses. In fact, the second dose of α -GalCer yielded peptide-specific CTL responses. The percentage of specific lysis between the two groups is statistically significant ($P < 0.05$). Steinman's group showed that *ex vivo* α -GalCer pulsed DC can be used to overcome the NKT cell anergy which results from repeated parenteral dosing with α -GalCer [8, 21]. However, our studies showed that administration of up to 3 doses of free α -GalCer via the intranasal or oral route does not hinder the induction of antigen-specific T-cell responses, and multiple doses are in fact beneficiary to induce antigen-specific T-cell responses in the various mucosal tissues. These results confirm and extend the similar studies by the Kang group where three intranasal doses of α -GalCer ad-mixed with either the OVA protein or the HA protein from the influenza virus were effective in priming strong antigen-specific systemic and mucosal immune responses to tumor and viral antigens [7]. Further studies are needed to determine the kinetics of the induction of mucosal immune responses after intranasal and oral routes, and how they differ from those observed after intravenous immunization, particularly with respect to the APCs.

In the present study, α -GalCer was evaluated for its ability to act as a mucosal adjuvant when administered either intranasally or orally with peptide antigens. Both Intranasal immunization and oral immunization of peptide antigens ad-mixed with α -GalCer induced antigen-specific CTL responses systemically and antigen-specific IFN- γ producing cells both systemically and in various mucosal compartments including the gut lymph nodes. These mucosal immunization routes are attractive because they eliminate the need for sterile syringes and needles, and also are painless to administer making it attractive for mass vaccinations against HIV in the under-developed world [22].

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Fig. 1. Intranasal immunization using a-GalCer as an adjuvant

Balb/C mice were immunized with a mixture of a-GalCer (2ug) and the HIV-1 envelope peptide R15K (100ug) and five days later the mice were sacrificed to collect different tissues as shown in the immunization scheme (A). Single cell preparations from spleens were analyzed for antigen-specific cytolytic activity by the standard chromium-release assay employing the syngeneic P815 target cells pulsed in vitro with either the immunizing peptide (R15K) or the CTL epitope peptide (R10I), at different effector to target cell ratios (B). The % specific lysis values shown are adjusted to un-pulsed P815 cells. Cells harvested from spleen and MLN were also analyzed for the numbers of IFN- γ producing cells using the standard cytokine ELISpot assay by incubating for 48 hours with media alone (Black), the immunizing peptide R15K (Grey), or the specific CTL epitope R10I (White). The numbers of IFN- γ producing cells were enumerated from the total 2×10^5 input cells. Data shown are an average of three different experiments.

Fig. 2. Repeated intranasal immunization with α -GalCer and R15K induces efficient antigen-specific cellular immune responses

Groups of Balb/C mice were immunized with either two doses (B, C) or three doses (D, E) of the mixture of α -GalCer (2 μ g) and R15K peptide (100 μ g) and cells were collected from the spleen and mucosal tissues (Immunization scheme in A). Cytolytic activity was assayed in the spleen cells using syngeneic P815 target cells pulsed in vitro with the immunizing R15K peptide and the CTL epitope peptide R10I (B and D) and adjusted by subtracting the values for un-pulsed target cells. The IFN- γ producing cells were enumerated within the

spleen and MLN by cytokine ELISpot assay (C and E). Data shown are average values from three separate experiments.

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Fig. 3. Repeated oral Immunization with aGalCer and R15K is effective in priming antigen-specific cellular immune responses

Groups of Balb/C mice were immunized with either two doses (B and C) or three doses (D and E), of a mixture of α -GalCer (2 μ g) and R15K (100 μ g) and cells collected from the spleen were analyzed for cytolytic activity (B and D) against syngeneic P815 target cells pulsed with the CTL epitope peptide R10I; IFN- γ producing cells were enumerated in the spleen and mesenteric lymph nodes (MLN) by the cytokine ELISpot assay (C and E). Data shown are average values from three separate experiments corrected for background values with un-pulsed target cells for the cytolytic assay.

Fig. 4. Induction of cognate protein-specific CTL responses after oral or intranasal immunization with the mixture of a-GalCer and R15K

Groups of Balb/C mice were immunized with two doses of a-GalCer (2ug) and the HIV envelope peptide R15K (100ug) by either the oral or intranasal (IN) route (scheme as shown in A). Spleen cells were re-stimulated for 5 days with the immunizing peptide and the cytolytic activity determined using the syngeneic P815 target cells infected overnight with either a control vaccinia virus (vSC8) or a recombinant vaccinia virus expressing the HIV envelop protein gp160 (vPE16) employing different effector to target cell ratios as shown (B). Data shown are average values from three separate experiments, corrected for background values with the vSC8 infected target cells.

Fig. 5. Repeated mucosal immunization using the α -GalCer adjuvant does not adversely affect induction of antigen-specific CTL responses

Groups of Balb/C mice were immunized by the oral or intranasal route with a mixture of α -GalCer (2 μ g) and R15K (100 μ g) followed by a second immunization with either the R15K peptide (100 μ g) alone or along with α -GalCer (2 μ g) on day 5 before sacrificing on day 10 (scheme as shown in A). Spleen cells collected from mice immunized by the oral or intranasal route were re-stimulated for 5 days with the peptide and analyzed for cytolytic activity using syngeneic P815 target cells pulsed in vitro with the R15K peptide (B). Data shown are average values after subtracting background values from un-pulsed target cells from three separate experiments.