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Intravenous Repeated Application of Low Dose α-Galatosylceramide Boosts IFN-γ Production in BALB/c Mice

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ABSTRACT

The aim of this study is to illustrate the relationship between α-Galatosylceramide (α-GalCer) dose and α-GalCer induced anergy of immune system in vivo. α-GalCer, a glycolipid and a widely studied immune modulator extracted from a marine sponge, shows its effect in vivo via presentation on CD1d molecule, MHC I like molecule bound glycolipid antigens, to NKT cells, a lymphocyte has both TCR and NK1.1, marker of T cells and NK cells respectively. The contradictory reports about α-GalCer are focused on two opposite axes of immune system: cancer and autoimmunity, failure and exaggeration of Th1 type immune response, respectively. In this study, the relation between the α-GalCer dose and the anergy was considered to clarify this dilemma. The groups of BALB/c mice were injected with various titer of α-GalCer intravenous way repeatedly and serum level of IFN-γ was measured with mouse IFN-γ ELISA. The Dramatic difference was found between groups after 2nd application of α-GalCer: Low dose α-GalCer boosted IFN-γ; however, high dose, usual dose cited in literature, failed to induce IFN-γ production. Besides, low concentrations of _-GalCer succeeded considerable amount of IFN-γ in the serum after priming. This results show that minute level of α-GalCer can be recognized by NKT cells, and repeated in vivo application of low dose α-GalCer can improve Th1 capabilities of the immune system.

Keywords: NKT cells, Galactosylceramide, anergy, Th1

ÖZET

Düşük Doz α-Galaktozilseramid'in İntrevenöz Tekrarlanan Uygulaması BALB/c Farelerinde IFN-γ Üretimini Artırır

Bu çalışmanın amacı, α-Galaktozilseramid (α-GalCer) dozu ile α-GalCer tarafından uyarılan immün sistem anerjisi arasındaki ilişkiyi resmetmektir. α-GalCer (bir glikolipid ve bir deniz süngerinden elde edilmiş genişçe çalışılmış bir immün modülatör) etkisini CD1d molekülü (MHC I'e benzeyen glikolipid antijenlere bağlanan molekül) üzerinde NKT hücrelerine (T hücre belirteci olan TCR ve NK hücre belirteci olan NK1.1'in her ikisine birden sahip olan bir lenfosit) sunulmasıyla gösterir. α-GalCer hakındaki birbiriyle çelişkili raporlar immun sistemin iki karşıt ekseni üzerine odaklanmıştır: kanser ve otoimmünite (sırasıyla, Th1 tip immün cevabın iflası ve aşırılığı). Bu çalışmada, bu ikilemi aydınlığa kavuşturmak için, α-GalCer dozu ve anerji arasınaki ilişki göz önüne alındı. BALB/c fare gruplarına tekrarla _-GalCer 'in birçok titresi intravenöz yolla enjekte edildi ve serum IFN-γ seviyesi fare IFN-γ ELISA ile ölçüldü. İkinci uygulamadan sonra gruplar arasında dramatik fark bulundu: Düşük doz α-GalCer IFN-γ'ı arttırdı; oysa, yüksek doz (literatürde belirtilen herzamanki doz) üretimini uyarmayı başaramdı. Bununla beraber, ilk uygulamadan sonra α-GalCer'in düşük konsantrasyonları serumda dikkate değer miktarda IFN-γ'ı başardı. Bu sonuçlar göteriyor ki; α-GalCer'in önemsiz miktarları NKT hücreleri tarafından tanınabiliyor ve düşük doz α-GalCer'in tekrar edilen in vivo uygulamaları immün sistemin Th1 yeteneklerini iyileştiriyor.

Anahtar Kelimeler: NKT hücreleri, Galaktozilseramit, anerji, Th1

INTRODUCTION

α-Galatosylceramide (α-GalCer) was isolated first time from the marine sponge, Agelas mauritianus, by the Pharmaceutical Division of the Kirin Brewery Company during a screening process of agelasphins, antitumour and immunostimulatory cerebrosides.[1-4] The chemical composition of α-GalCer resembles mammalian glycolipids; yet, it does not belong to human or mice. On the one hand, α-GalCer has a well documented antimetastatic and antitumor potency in experimental tumor models of mice.[5-9] On the other hand, there are many reports that show its ameliorating effects on autoimmune disease models such as, type 1 diabetes, experimental allergic encephalomyelitis, arthritis, systemic lupus erythematosus, inflammatory colitis, and Graves' thyroiditis.[10-16] α-GalCer is like a "double-edged sword" drives the immune system response to diametrically opposite sites, Th1 and Th2, simultaneously.[17,18] Not only that, but also the "cytokine storm", appears after in vivo α-GalCer application, composes of IL-4, IFN-γ, GM-CSF, TNF-α, IL-2, IL-10, IL-13, and, TGF-β, members of both Th1 and Th2.[17,19] To deviate the immune response to either Th1 or Th2, in other words to put into or protect from anergy of immune system, α-GalCer analogue with different chemical structure was developed, and α-GalCer application protocol was modified.[20-24] In short, α-GalCer as an immune modulator has a paradoxical immune response itself and needs refinement of application protocol or chemical composition.

Natural Killer T cells (NKT cells) are the only responsible cell type of α-GalCer restricted immune reactivity in mice, rats and humans. α-GalCer is recognized by NKT cells via presentation on CD1d, a MHC-like molecule similar to the other CD1 family members specialized for presenting self and foreign lipid antigens to T lymphocytes.[25] NKT cells, believed to be the bridge between innate and adaptive immunity, are a separate lineage of T lymphocytes co-express both NK1.1 and TCR, markers of NK cells and T cells respectively.[19,26-28] Consequent to initial α-GalCer exposure, NKT cells secrete IL-4 and IFN-γ cytokines rapidly in massive amounts. Moreover, following α-GalCer recognition NKT cells proliferate and show phenotypic alterations like transient TCR down regulation and sustained NK1.1 downregulation.[29] NKT cells enter an unresponsive state, what is also called "anergy of NKT cells", reflected with lack of IFN-γ and low IL-4 in restimulation, after α-GalCer priming.[21]

In this study it is questioned that the anergic state of immune system created by α-GalCer can be overcome with modification of the α-GalCer dose. Here, in other words, it is simply hypothesized that commonly used level of α-GalCer in vivo experiments, 2-5 μg, develop unresponsive NKT cells to restimulation, but not lower level of α-GalCer.

MATERIALS and METHODS

Mice: BALB/c mice are bred in our laboratories. 8 weeks-old mice were used for this study.

Reagents: α-GalCer was provided kindly by Dr. Uenaka Akiko, from Okayama University, as 2mg/ml solution. It was diluted to indicated concentrations in RPMI.

Sample preparation: 4 group of mice (n= 2) were constituted according to given α-GalCer amount, 2000 ng, 200 ng, 20 ng and 2 ng, per mouse. Than, indicated dose was given i.v. twice with 10 days interval. for the analyses. After every injection, blood samples of mice were obtained via bleeding of tail at 0th, 6th, 12th, 24th, and 48th hours. After that, serums are kept in -80^{0}C until use for ELISA assay.

Enzyme-linked immunosorbent assay (ELISA): Blood is obtained from tail by bleeding. ELISA plates pre-coated with anti-mouse IFN-γ mAb, R4-6A2, are blocked with 5% FCS in PBS for 2 h at 37^{0}C and washed with 0.05% Tween 20 in PBS. The serum (1:100) is transferred to the plates and incubated for 1 h and then rabbit anti-mouse IFN-γ (1:1600) is added and incubated for 1 h at 37^{0}C. After washing, HRP-conjugated goat antirabbit antibody (MBL, Nagoya, Japan) (1:2000) is added to the wells and the plates were incubated for 1 h at 37^{0}C. For color development, OPDA and H_2O_2 in citrate buffer were added. The reaction is stopped with 6 N H_2SO_4. Absorbance at 490 nm is read with a microplate reader (Benchmark; Bio-Rad, Hercules, CA,USA).

Statistics: Paired t-test is used to asses the significance of results. P-values lower than 0.05 accepted as significant.

RESULTS

Both in vivo and in vitro, NKT cells recognize α-Galcer, a foreign antigen to human and mice.[19] Int-

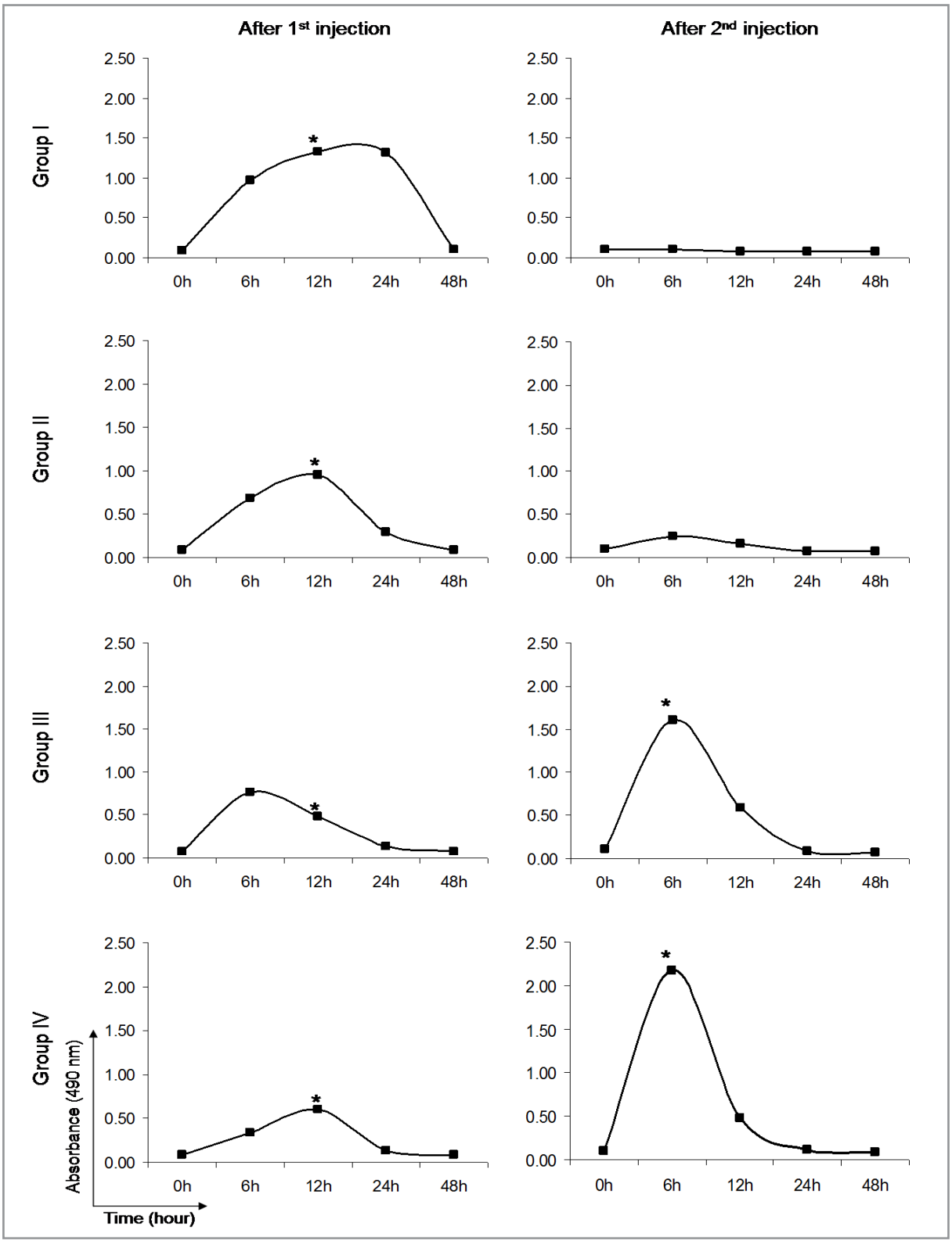

Figure1. Low dose of α-GalCer boost IFN-γ release of NKT cells (mouse IFN-γ ELISA). BALB/c mice are injected intravenously with 2000 ng, 200 ng, 20 ng or 2 ng of α-GalCer, group I, II, III and IV (n= 2) respectively. Than, blood samples are collected by tail bleeding at 0h, 6h, 12h, 24h, and 48h after 1st and 2nd injections. IFN-γ levels in serums are defined with duplicated mouse IFN-γ ELISA. This experiment is repeated twice. Absorbance value shows the mean of two mice. (*Student t-test is used ($p \leq 0.0001$))

ravenous injection α-Galcer urges robust cytokine secretion of NKT cells; nonetheless, they become inactive to a second intravenous treatment of α-Galcer. Eventually, anergy of NKT cells attracted our attention to the possibility of α-Galcer dose could be higher than normal for i.v. application. IFN-γ was detected in all samples from primed groups and the level was directly related the applied dose of α-Galcer (Figure 1). As expected, IFN-γ was not detected in the serum gathered from the 2 μg α-Galcer re-injected group of mice (Figure 1). However, high amount of IFN-γ was shown in the samples collected from the 20 and 2 ng α-Galcer re-injected groups of mice, surprisingly (figure 1). What is more, the IFN-γ production was enhanced apparently in the latter groups. This result indicates two points: First, anergic state of NKT cells are directly related to the concentration of α-Galcer. In other words, repeated application of 2 ng and 20 ng α-Galcer can still activate NKT cells in vivo. Second, such remarkably small amount of α-Galcer, also a foreign antigen, can be recognized by NKT cells.

DISCUSSION

NKT cells are the main source of the IFN-γ which is important for maturation of antigen presenting cells and activation of both Natural Killer cells and cytotoxic T cells.[30] Today, NKT cells are believed to be one of the immune regulatory cell types.[31] NKT cell also is attributed to many auto immune and oncological diseases in human and animal models. α-GalCer is a potent activator of NKT cells and drive them to immediate production of IFN-γ and IL-4, main cytokines of th1 and th2 mediated immune responses, respectively. In this study, first time, in vivo data of priming and re-stimulation with various α-GalCer doses is presented. Moreover, first time, it is shown that much lower amounts of α-GalCer than regularly used amount lead the NKT cells IFN-γ production after both priming and re-stimulation. In fact, re-stimulation with low doses of α-GalCer boost IFN-γ production of NKT cells.

NKT cell is cytokine station of immune system which can fuel dominant helper response,th1 or th2, at the moment of activation. According to scene presented in this paper, repeated stimulation of NKT cells with low dose α-GalCer can provide more IFN-γ to improve tumor antigen presentation with maturation of antigen presenting cells, and activate Natural Killer cells and Cytotoxic T cells (CD8 T cells) better than high dose. Unfortunately, it is difficult to estimate the ultimate direction of th1 mediated autoimmune diseases in our experimental settings because of lack of IL-4 data at low dose of α-Galcer. However, it seems that sustained IFN-γ presence can exacerbate autoimmune diseases in low doses of α-GalCer injection.

Why does re-stimulation with high dose α-GalCer not activate high dose α-GalCer primed NKT cells? It is reported that after priming with high dose α-GalCer, NKT cells first down regulate NK1.1 and TCR cell surface expression, and then up regulate their cell surface expression in few days, finally number of NKT cells expand.[32,33] Logically, under these circumstances it is expected that larger number of fully intact NKT cell produces much more IFN-γ; however, it does not surprisingly. One explanation of this may be high dose α-GalCer develops anergy in NKT cells.[34] The other explanation of this may be high dose α-GalCer can polarize NKT cells toward th2 cytokine synthesis.[35,36] The third explanation of this can be IFN-γ induced Qa-1b expression can inhibit reactivation of α-GalCer primed NKT cell.[37] The last explanation can be applied to present finding as initial low dose α-GalCer can not succeed the systemic IFN-γ threshold level to trigger Qa-1b expression dependent inhibition. In such case, 2nd treatment will start the inhibitory mechanism due to boosted IFN-γ. To clarify this, a 3rd shut of α-GalCer is needed; however, we do not have this data at the moment.

Various approaches are reported in literature including DC loading, intradermal delivery of α-GalCer, modification of α-GalCer structure and PD1/PDL blockade to weaken and halt the unresponsive state of NKT cells occurring in α-GalCer deployed experimental treatment methods.[20,21,38,39] Here, another simple way, decreasing the amount of α-GalCer, is shown to modulate α-GalCer restricted immune response for in vivo applications.

In addition, it is note worthy that this report discloses the tiny level of α-GalCer can reactivate NKT cells and drive them to secrete cytokine, in this case IFN-γ. It seems that NKT cells are primed with low doses of glycolipids, expanded and reactivated

like traditional T cells. If our conclusion is valid, it should be reconsidered the concentration of glycolipid antigens to understand NKT cell biology better and define their possibly new self and non-self antigen repertoire.

In conclusion, in vivo α-GalCer dose influences heavily the following immune response outcome: high dose suppresses and low dose enhances.

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