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Original Research

Polyclonal Antibody Production Against Hapten-Structured KDN Molecule by Using Different Adjuvants Alternative to Freund's Adjuvant

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ABSTRACT

Objective: KDN (2-keto-3-deoxy-D-glycero-D-galacto-nononic acid), a member of the sialic acid family, is a hapten-structured low-molecular-weight monosaccharide on the cell membrane, which cannot induce immune responses without a carrier protein. Since it is over-expressed on the cancerous cells' membrane, it is thought to be a great target molecule for anti-cancer treatments. The aim of this study is to obtain high titersof the anti-KDN polyclonal antibody response without using any carrier protein against the hapten-structured KDN molecule alternative to Freund's adjuvants.

Methods: Montanide™ ISA 61 VG, a water-in-oil adjuvant; ISA 201 VG, a water-in-oil-in-water emulsion adjuvant; and IMS 1313 VG NPR, an aqueous-dispersion-based nanoparticle (50-200 nm) microemulsion adjuvant; and Freund's adjuvant were used as anti-KDN antibody response stimulators. FourBALB/c mice were used for each adjuvant group, and immunization was performed at eight different time points. Anti-KDN antibody levels induced after each immunization with different adjuvants were detected with indirect enzyme-linked immunosorbent assay.

Results: The adjuvant efficiency of Montanide™ ISA 61 VG water in oil adjuvant was 1.4 times higher than in Freund's adjuvant ($p<0.0001$), with a maximum anti-KDN level on Day 83.

Conclusion: It's shown that without any carrier protein conjugation molecules such as hapten-structured KDN, higher amount anti-KDN antibody titres could be obtained by using a more safe and effective Montanide™ ISA 61 VG water-in-oil adjuvant as an alternative to Freund's adjuvants. In this regard, it may be possible to produce high-antibody titers without using any carrier molecule, especially when commercial large scale monoclonal antibodies are desired to be produced against haptens as therapeutic approaches.

Keywords: KDN (2-keto-3-deoxy-D-glycero-D-galacto-nonionic acid), polyclonal antibody, ELISA

INTRODUCTION

KDN, an abbreviation for 2-keto-3-deoxy-D-glycero-D-galacto-nononic acid, was discovered in 1986 and is found in almost all glycoconjugates containing glycolipids, glycoproteins, and capsular polysaccharides. It has been reported that KDN has higher expression levels in fetal red blood cells than in adult red blood cells, as well as in ovarian tumor tissue cells, which have a higher KDN expression in comparison with normal ovarian tissue (1).

2-keto-3-deoxy-D-glycero-D-galacto-nononic acid, a member of the sialic acid family has distinctive properties such as insensitivity to various sialidases and the association of N-acetyl group with the hydroxyl group of NeuAc (N acetylneuraminic acid) has been shown to be related to human cancers (2-3). The synthesis of KDN by organic routes makes it possible to become an important target for anti-cancer therapies (4). Ketocidic bonds in the diamine sialic acid type KDN cause resistance to bacterial and animal sialidases and protect against bacterial and viral attacks (1). In addition, KDN exhibits metastasis and anti-Alzheimer activity as it stops the glycolipid synthesis as an immunoregulator (5). Due to these features, studies on the use of KDN as an early warning signal of disease or in case of disease recurrence have been reinforced (6). In addition, the KDN expression level is elevated in ovarian adenocarcinomas and is associated with metastasis (7).

2-keto-3-deoxy-D-glycero-D-galacto-nononic acid is a relatively low-molecular-weight molecule of hapten character (4). Haptens are small molecules capable of reacting with antibodies that are specific immune-response products, having antigenicity, but not immunogenicity alone, capable of producing an immunological response only after binding with a carrier macromolecule (e.g., protein) (2, 8, 9). In general, it is thought that only large molecules, infectious agents, and insoluble foreign substances cause immunological reactions in the body (10). For the application of vaccine

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production, the use of protein carriers is preferred in the conjugation of haptens (11). However, in the polyclonal or monoclonal antibody production, the antigen is expected to be pure enough to induce a specific immune response. In addition, the purification steps become difficult because of the carrier protein. Although in the laboratory scale production, the purification problem can be ignored, in large-scale production, the cost of purification steps will increase the overall cost of the antibody production process (11). In this context, the ultimate goal of the study is to find an alternative adjuvant to the protein conjugates for hapten-structured KDN.

Freund's adjuvants are emulsion adjuvants that are the most commonly used the oil-in-water emulsion for immunization studies (12, 13). The main disadvantages of them are toxicity, the formation of granulomas, inflammation, and lesions at the vaccination site. In this regard, the development of new adjuvants as an alternative to Freund's adjuvants is on the agenda(14, 15). Montanide™ adjuvants are non-toxic systems, optimized to increase the vaccine efficacy, formulation stability, and also to minimize side effects. There are commercialized vaccine registries using Montanide™ adjuvants, and they have been shown to be reliable in scientific studies(15-17; www.seppic.com/montanide-range). Montanide™ ISA 61 VG is a water-in-oil adjuvant; ISA 201 VG is a water-in-oil-in-water emulsion adjuvant, and IMS 1313 VG NPR is an aqueous dispersion based on nanoparticle(50-200 nm) microemulsion adjuvant with immune stimulators.

Although the Freund's adjuvants are the most used adjuvant systems in animal experiments, it is ethically controversial to use them due to their side effects (local acute or chronic inflammation, abscess at the injection site, a permanent nodule, wound, or lymphadenopathy)given the 3R rule (replacement, reduction, and refinement) in the use of experimental animals. Thus, using the Montanide™ adjuvants also address the same ethical issue of using Freund's adjuvants.

The aim of this study is to obtain a high titer anti-KDN polyclonal antibody response using safer and more efficient adjuvant systems that are alternative to Freund's adjuvants without using any carrier protein against the hapten-structured KDN molecule.

METHODS

Chemicals and Experimental Animals

Montanide™ ISA 61 VG (Seppic, France), Montanide™ ISA 201 VG (Seppic, France), and Montanide™ IMS 1313 VG NPR (Seppic, France) adjuvants were obtained from (Seppic Turkey, YILBAK Tic. A.Ş.). KDN (Cat no: 60714), Freund's complete adjuvant (FCA; Cat no: F5881) and Freund's incomplete adjuvant (FICA; Cat no: F5506), NaCl, KCl, bovine serum albumin (BSA),o-phenylenediaminedihydrochloride (OPD), and citric acid were purchased from Sigma, USA; horseradish peroxidase (HRP)-labeled anti-mouse IgG was from Thermo Scientific, USA and $Na_2HPO_4.2H_2O$, Tween-20 and H_2O_2 from Merck, Germany. Six- to eight-week-old BALB/c mice were purchased from Uludağ University Research Center for Breeding Test Animals (DEHYUAM). This study has been approved by Ege University's Animal Experiments Local Ethics Committee on 25/11/2015 with an approval number of 2015-084.

Immunization with KDN

Immunization studies were carried out at the Ege University, Drug Development and Pharmacokinetics Research and Application Center (ARGEFAR) pre-clinical development unit. Five experimental groups including four mice in each group-Montanide™ ISA 61 VG, Montanide™ ISA 201 VG, and Montanide™ IMS 1313 VG NPR adjuvants, Freund's adjuvants, and the control group that were not immunized with KDN-were used. In the Freund's adjuvant group, FCA was used in the first immunization, and FICA was used in the following immunizations. Before the first immunization, blood was drawn from the tail of all mice as 0.2-0.5 mL/mouse to obtain the blood serum. In the first immunization, 100 µg KDN antigen in 100 µL saline was injected subcutaneously with 100 µL adjuvant in a total volume of 200 µL for each mouse. In the second immunization on Day 14, mice were injected intraperitoneally with 50 µg KDN/100 µL saline and 100 µL adjuvant in a total volume of 200 µL. The third immunization was performed on Day 28, the fourth immunization on Day 55, the fifth immunization on Day 72, the sixth immunization on Day 84, the seventh on Day 98, and the eighth day on Day 112, subcutaneously with 50 µg KDN/100 µL saline and 100 µL adjuvant in a total volume of 200 µL. For the indirect enzyme-linked immunosorbent assay (ELISA), blood was drawn from the tail after the third immunization at the 41st, 69th, and 83rd days, respectively, and from the heart on the 125th day.

Blood Sample Collection and Serum Separation

Primarily, mice tails were treated with warm water to make the vein more visible. When the vein became clearly visible, the tail was wiped with alcohol, and the intracath was introduced into the vein to collect 0.2- 0.5 mL of blood. For bloodletting from the heart, ketamine (60-90 mg/kg) and xylazine (4-7 mg/mouse) were administered to mice, and they were euthanized by cervical dislocation. All blood was collected as much as possible by injecting into the heart ventricle. Blood samples were incubated overnight at +4^{0}C. And were centrifuged at 3000 rpm for 10 minutes to separation. After centrifugation, the supernatant (serum) was removed and stored at -20°C for indirect ELISA.

Indirect ELISA to determine anti-KDN antibody titer

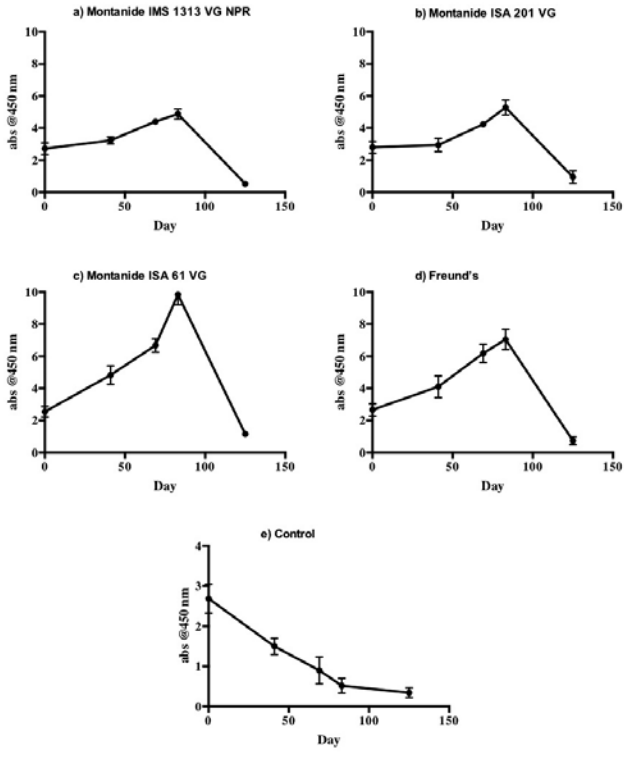
In indirect ELISA, the ≥99.0% thin-layer-chromatography-grade KDN molecule was used to coat the plates. Briefly, the KDN protein was diluted to a final concentration of 175 ng/well in phosphate buffered saline (PBS), added 100 µL into the ELISA plates, and incubated overnight at 37°C. The wells were spilled, and 100 µL blocking solution consisting of 0.5% (w/v) BSA in 0.1 M PBS was added to the wells and incubated for 1 hour at room temperature. The plates were washed three times with 150 µL TPBS (0.1 M PBS with 0.1% (v/v) Tween-20). The 100 µL blood serum samples and controls were added as the primary antibody, and plates were incubated for 1 hour at room temperature. The plates were washed three times with 150 µL tween phosphate buffered saline (TPBS). A 100 µL diluted conjugated secondary antibody (anti-mouse IgG conjugated with HRP was diluted in TPBS) was added, and plates were incubated 1 hour at room temperature. The plates were washed three times with 150 µL TPBS. 200 µLof substrate solution (30 mg of OPD was dissolved

Table 1. ELISA results of antibody titer against KDN obtained after immunization with different adjuvants

	Absorbance @450 nm				
Day	Montanide™ IMS 1313 VG NPR Adjuvant	Montanide™ ISA 201 VG Adjuvant	Montanide™ ISA 61 VG Adjuvant	Freund's Adjuvant	Control (without immunization with KDN)
0	2.725±0.372	2.804±0.370	2.548±0.326	2.660±0.380	2.684±0.362
41	3.240±0.219	2.948±0.409	4.833±0.590	4.106±0.679	1.495±0.202
69	4.416±0.183	4.236±0.174	6.678±0.420	6.175±0.577	0.900±0.339
83	4.881±0.318	5.283±0.466	9.848±0.630	7.044±0.630	0.515±0.183
125	0.536±0.169	0.946±0.399	1.156±0.095	0.737±0.238	0.340±0.124

Montanide™ ISA 61 VG: water-in-oil adjuvant; Montanide™ ISA 201 VG: water-in-oil-in-water emulsion adjuvant; Montanide™ IMS 1313 VG NPR: aqueous dispersion based on nanoparticle (50-200 nm) micro emulsion adjuvant

Figure 1. Changes in anti-KDN pAb absorbance values of blood serum obtained after immunization using different adjuvants with respect to days: immunization with Montanide™ IMS 1313 VG NPR, which is an aqueous dispersion based on nanoparticle (50-200 nm) micro emulsion adjuvant (a); immunization with the Montanide™ ISA 201 VG water-in-oil-in-water emulsion adjuvant (b); immunization with the Montanide™ ISA 61 VGa water-in-oil adjuvant (c); immunization with Freund's adjuvant (d); control group without immunization with KDN (e)



in 75 mL 0.05 M of the phosphate-citrate buffer, and 30 µL fresh 30% [v/v] H2O2 was added immediately prior to use) was added, and the plates were incubated for 30 minutes in dark at room temperature. After 30 minutes, the absorbance was recorded at 450 nm wavelength with a UV-visible spectrophotometer (SpectraMax 190, VersaMax, USA).

Statistical Analysis

The obtained data were evaluated in the two-way variance analysis (two-way ANOVA) with ±95% confidence interval using GraphPad Prism 6.0e, taking into account the days of immunization and the different adjuvant types.

RESULTS

The anti-KDN polyclonal antibody level according to the ELISA results obtained from Montanide™ ISA 61 VG, Montanide™ ISA 201 VG, Montanide™ IMS 1313 VG NPR, Freund's adjuvants, and the control group on Days 0, 41, 69, 83, and 125 is shown in the Table1.

According to Figure 1, all groups reached the highest level of anti-KDN polyclonal antibody on Day 83. With Freund's adjuvant, the highest absorbance was 7,044±0,630; with the Montanide™ adjuvants including IMS 1313 VG NPR, ISA 201 VG and ISA 61 VG; 4.881±0.318, 5.283±0.466, and 9.848±0.630 absorbances were obtained respectively after the last immunization. In the control group, which was not immunized with KDN, the absorbance decreased on a daily basis (Figure 1).

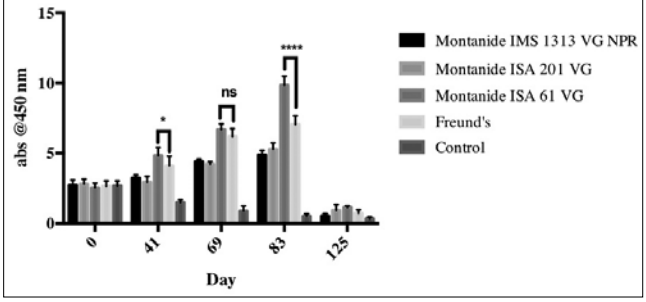
When evaluating the resulting anti-KDN titers, the Freund's adjuvant was taken as the basis and compared with different Montanide™ adjuvants whose efficacy in this study was to be compared on the same graph in Figure 2.

According to Figure 2, there was no statistically significant difference ($p>0.05$) between the blood samples taken from all groups before immunization (Day 0). Based on the results of the 41st, 69th, and 83rd days ELISA, it was determined that the polyclonal antibody titer obtained after using the Montanide™ IMS 1313 VG NPR and Montanide™ ISA 201 VG adjuvants was lower than the polyclonal antibody titer obtained with Freund's adjuvant. When the Montanide™ ISA 61 VG and Freund's adjuvants were compared, the level of the anti-KDN polyclonal antibody titer obtained using the Montanide™ ISA 61 VG adjuvant was higher at last three immunization points, especially 1.4 times higher on the Day 83 ($p<0.0001$).

DISCUSSION

2-keto-3-deoxy-D-glycero-D-galacto-nononic acid is a sialic acid family specific monosaccharide found on the cell membrane.

Figure 2. Comparative study of the Montanide™ adjuvant groups activities according to Freund's group: Montanide™ ISA 61 VG is a water-in-oil adjuvant; Montanide™ ISA 201 VG is a water-in-oil-in-water emulsion adjuvant; Montanide™ IMS 1313 VG NPR is an aqueous dispersion based on nanoparticle (50-200 nm) micro emulsion adjuvant. *: p<0.05; ****: p<0.0001; ns: non-significant p>0.05



It has been discovered for the first time in a patient with ovarian cancer that the level of free KDN molecules is high; thus, it is thought to be an antigen that promotes positive malignancy with cancer development, because it is generally exaggerated at low levels in mammalian cells (1, 4, 6, 18). After that, several studies showed that the KDN expression is high in cancerous cells, and KDN expression is associated with tumoral, nodal, and metastatic differentiation of tumors (5, 6, 18). It has been found that while the hypoxic environment enhances the invasiveness and metastatic ability of cancer cells (e.g., HeLa; adenocarcinoma, CaCo-2; colorectal adenocarcinoma and LS174T Dukes' type B; colorectal adenocarcinoma), it also induces the production of KDN in these cells by creating changes in the sialic acid metabolism (4, 6).

With haptens, like with KDN, the immunological response is usually achieved by chemical modification with the hapten-carrier complex (e.g., BSA-bovine serum albumin) and modifications to introduce active groups. On this account, artificial antigens can be obtained and used to stimulate B-cell proliferation and T-cell differentiation to produce antibodies against the hapten molecules (10, 11, 19). BSA is the most common carrier protein in the macromolecular, stable structure of the single polypeptide chain without carbohydrates, which is preferred for haptens in the conjugation reactions due to various functional groups carried by the structure in the antibody formation(10, 20, 21).

The name adjuvants, one of the conjugates used to increase the immunity of the haptens, is derived from the Latin word adjuvare, which means help or healing (12). To be effective, adjuvants must affect dendritic and macrophage cells, which are antigen-presenting cells in the natural immune system, to stimulate T and B lymphocytes in the living organism (11, 22). Although there are many types of adjuvants, oil-water-based emulsions and micro-nanoparticles are classified as adjuvants for antigen transport, and cytokines and saponins are classified as adjuvants for immune stimulators according to their mechanism of action (12, 22).

Freund's adjuvants are the most commonly used systems in immunization studies, called incomplete Freund's adjuvant (FICA) when prepared with non-metabolic oils and complete Freund's adjuvant (FCA) when prepared with dead Mycobacterium tuberculosis (12). Freund's adjuvants are designed to provide a sustained release of the antigen needed to stimulate a strong and lasting immune response (13). However, the use of these adjuvants for antibody production usually causes very severe lesions in experimental animals (14). For this reason, many regulatory and supervisory rules for licensing have been rearranged to limit the use of such adjuvants. In this regard, the development of new adjuvants has come to the agenda as an alternative to Freund's adjuvants (15).

The Montanide™ adjuvants are systems that have been scientifically proven to be reliable and available with commercialized vaccine products already on the market. They are optimized to increase vaccine efficacy and formulation stability and also to minimize side effects. These adjuvants usually include a surfactant system: in general, emulsions such as water-in-oil, water-in-oil-in-water, oil-in-water, and in nanoparticulate form (15-17, www.seppic.com/montanide-range).

The Montanide™ ISA 61 VG used in this study is an adjuvant that provides a powerful cellular immune response in the form of water-in-oil emulsion, fortified with light mineral oils to enhance the Th1 response and production of IgG2, with the addition of mannitol and vegetable oleic acid, without carrying an animal component (23, 24; www.seppic.com/montanide-isa-w-o). The Montanide™ ISA 201 VG adjuvant is a water-in-oil-in-water emulsion of similar features to the Montanide ISA 61 VG adjuvant. It stimulates both humoral and cellular long immune response (23, 24; https://www.seppic.com/montanide-isa-w-o-w). The Montanide™ IMS 1313 VG NPR adjuvant is a formulation in the form of an aqueous dispersion based on nanoparticles (50-200 nm). It contains immunostimulatorygenerally recognized-as-safe substances, and it rapidly stimulates both humoral and cellular immunological response (25; www.seppic.com/montanide-ims).

Antigen-antibody interactions are important in ELISA, and antigen-antibody interfaces are primarily controlled by hydrophobic and electrostatic interactions (9). In this study, immunization was performed at eight-time points, the last one on the 125th day. The blood samples taken at regular intervals were tested by indirect ELISA to determine the level of antibody response to KDN. Freund's adjuvants, which are the most commonly used adjuvant systems in scientific studies (12, 13), and the alternative Montanide™ (ISA 201 VG, ISA 61 VG, and IMS 1313 VG NPR) adjuvants were compared statistically in this study by two-way ANOVA test. Among the obtained anti-KDN polyclonal antibody levels, the highest anti-KDN polyclonal antibody titer is obtained with the Montanide™ ISA 61 VG adjuvant, which is 1.4 times more than the antibody levels obtained withFreund's adjuvant (P<0.0001). The Montanide™ ISA 61 VG adjuvant is specially developed for studying low-immunogenicity antigens in the form of water-in-oil (23, 24), which makes it a candidate for working with haptens (Figure 2).

As Figure 2 indicates, the absorbance values of anti-KDN polyclonal antibody were measured at a certain level in all adjuvant groups including the control group on the 0th day due to the

fact that KDN is generally excreted at low levels in normal life span in mammalian cells and these values are not statistically different from each other (p>0.05) (5, 6). Thereafter, it was observed that the anti-KDN antibody level increased by following immunizations, reached the maximum value on day 83rd, and immunity was decreased by age due to the immunological senescence (1, 5, 26). In the control group in which no KDN protein was given, it was determined that there was a decrease in the antibody level during lifetime from this cause (Figure 2). İbrahim et al. (24) performed immunization experiments in mice with the Montanide™ ISA 201, 206, 61 and 50 VG adjuvants for the foot-and-mouth disease vaccine development studies. Antibody titers were compared by ELISA in blood samples collected during the 20-week trial period. When the Montanide™ ISA 201 and 61 VG adjuvants were compared, it was also reported that the highest antibody titer was achieved with Montanide ISA 61 VG, and when the time-varying antibody titers were examined, the titers of antibodies were increased until the 10th week and started to decrease since then with aging due to the immunological senescence (24).

It has been stated that low-molecular-weight hapten-structured carbohydrates such as KDN can only bind to the receptors present on the surface of B lymphocytes, whereas they cannot activate the helper T cells and thus cannot achieve an antibody response against them (8). For this reason, it is stated that suitable carrier protein conjugates should be used (9). However, this study showed that the desired antibody response could be obtained using the Montanide™ ISA 61 VG water-in-oil adjuvant as an alternative to Freund's adjuvant without conjugating any carrier protein such as BSA to the KDN molecule.

CONCLUSION

It may be possible to produce high-antibody titers against hapten-structured proteins without using any protein carrier molecule when using alternative adjuvant systems. This is a promising result for a large-scale antibody production against haptens, especially for commercial therapeutic monoclonal antibody production process as means of antibody specificity and ease of purification steps.

Ethics Committee Approval: Ethics committee approval was received for this study from the animal experiments local ethics committee (EU-HADYEK) of Ege University (Approval Date: 25.11.2015; Approval No: 2015/084).

Informed Consent: N/A

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Conflict of Interest: The authors have no conflicts of interest to declare.

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