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Novel Detox Gel Depot sequesters β -Amyloid Peptides in a mouse model of Alzheimer's Disease

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Abstract

Alzheimer's Disease (AD), a debilitating neurodegenerative disease is caused by aggregation and accumulation of a 39–43 amino acid peptide (amyloid β or A β) in brain parenchyma and cerebrovasculature. The rational approach would be to use drugs that interfere with A β -A β interaction and disrupt polymerization. Peptide ligands capable of binding to the KLVFF (amino acids 16–20) region in the A β molecule have been investigated as possible drug candidates. Retro-inverse (RI) peptide of this pentapeptide, *ffvIk*, has been shown to bind artificial fibrils made from A β with moderate affinity. We hypothesized that a 'detox gel', which is synthesized by covalently linking a tetrameric version of RI peptide *ffvIk* to poly (ethylene glycol) polymer chains will act like a 'sink' to capture A β peptides from the surrounding environment. We previously demonstrated that this hypothesis works in an *in vitro* system. The present study extended this hypothesis to an *in vivo* mouse model of Alzheimer's Disease and determined the therapeutic effect of our detox gel. We injected detox gel subcutaneously to AD model mice and analyzed brain levels of A β -42 and improvement in memory parameters. The results showed a reduction of brain amyloid burden in detox gel treated mice. Memory parameters in the treated mice improved. No undesirable immune response was observed. The data strongly suggest that our detox gel can be used as an effective therapy to deplete brain A β levels. Considering recent abandonment of failed antibody based therapies, our detox gel appears to have the advantage of being a non-immune based therapy.

Keywords

Alzheimer's disease; Detox gel; retro-inverse peptide

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disease and is the most common cause of dementia among people age 65 or older. Although the etiology of AD is not completely understood, amyloid beta peptide (A β), the major component of senile plaques found in AD brains, is implicated in the pathogenesis of AD (Small et al., 2001). Amyloid beta aggregations as cytotoxic inclusions or amyloid-like plaques in the brain parenchyma and cerebrovasculature is perceived to be central to the pathogenesis of AD. Amyloid associated with Alzheimer's disease consists of thin fibrils of polymerized A β with an ordered β -sheet

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pattern in AD brains. The actual form of A β that causes the damage is still uncertain, but is most likely a small dimeric or higher oligomeric species with internal β -sheet structure (Selkoe, 1996). It has been well demonstrated that compounds intended to block β -sheet formation will inhibit the formation of both amyloid fibrils and smaller oligomers, and block A β toxicity toward neurons in *in vitro* and in animal models (Soto et al., 1998). A β binds to itself at residues 12–23. This region has been the basis for the synthesis of numerous small peptides as β -sheet blockers (Tjernberg et al., 1997). Therefore, the rational pharmacological approach would be to use drugs that interfere with A β -A β interaction and polymerization. Tjernberg *et al* (1996) deduced that the KLVFF segment in the truncated native A β sequence was of critical importance in the polymerization of amyloid fibril. Peptides that incorporate this sequence will bind A β and block fibril formation of A β -40/42. Peptides composed of D amino acids of this sequence were found to be as effective in preventing amyloid fibril formation and with increased protease resistance.

Retro-inversion technology involves reversing the primary sequence of a peptide and replacing L-amino acids with D-amino acids. This development in peptidomimetics, based on peptide-bond reversal and inversion of chirality has presented an increased possibility of designing superior peptide-based therapeutics (Kokkoni et al., 2006). Researchers found that the retro-inverso (RI) peptide, *ffvIk*, of the native sequence KLVFF binds artificial fibrils made from A β with moderate affinity and that increasing the copy number of peptide has an additive effect on its affinity for A β (Zhang et al., 2003).

A recent concept based on “peripheral sink” hypothesis was introduced for inhibiting the accumulation of plaques in the brain. This model is based on the finding that A β peptides can exit from the brain into the periphery (Strazielle et al., 2000). Thus, it might be possible to tip the balance toward removal rather than accumulation of A β in the CNS, based on the premise that A β equilibrates between the CNS and the periphery. Two publications tested this hypothesis. In an earlier study (DeMattos et al., 2002), monoclonal antibodies, which cannot cross the blood-brain barrier was injected intravenously into AD mice. In the latter study (Matsuoka et al., 2003), they injected a high-molecular-weight protein or a small lipid, both previously known to avidly bind to A β . Indeed, both reported depletion of plaques in the brain. Any of these 3 substances could possibly slow down or halt AD progression. Therefore, our therapeutic strategy and therapeutic reagent does not have to cross the BBB. Instead, the toxic A β peptides will cross the blood brain barrier (BBB), and will be captured in the periphery. This “sequestration” was tested using Congo red (Matsuoka et al., 2003). After 3 weeks, they reported that it did “not substantially alter brain A β load.” This failure suggested optimizing A β binding agents for greater affinity to A β peptides. Sagare et.al, on the contrary proved altering brain A β loads by using circulating lipoprotein receptors (sLRP) as a peripheral sink. In this study, circulating sLRP caused A β efflux from brain to plasma and sequestered the amyloid from plasma (Sagare et al., 2007).

We have developed a novel therapeutic combining the features of the A β binding agents with the properties of the subcutaneous PEG based hydrogel drug delivery and called it the “detox gel”. The principles of the peripheral sink approach to deplete the brain of toxic amyloid peptides using the detox gel was explored. We showed that these detox gels containing the tetrameric version of the *ffvIk* peptide bound A β -42 peptides effectively and irreversibly in an *in vitro* study (Sundaram et al., 2008). We showed that polyethylene glycol (PEG) conjugates bearing several copies of *ffvIk* can be useful as diagnostic and therapeutic agents for Alzheimer's disease. Essentially, these detox gels are made by covalently linking a particular RI peptide to poly(ethylene glycol) polymer chains. PEG was chosen as the building block of the new hydrogel-forming polymers. PEG is a non-toxic, non-immunogenic, highly water soluble polymer. Linking peptides (or proteins) to PEG is called

pegylation and this causes improved pharmacokinetics, increased circulation time and decreased toxicity

In the present study, we attempted to determine the therapeutic effect of our detox gel in AD model mice (APPSWE-Tg2546) (Hsiao et al., 1996). Results showed that our detox gel treatment led to a reduction of brain amyloid burden by while simultaneously improving memory parameters without any observable immune response.

MATERIALS AND METHODS

Peptides and other chemicals

RI-Tetramer peptide was synthesized at the Keck Foundation at Yale University, as described previously (Sundaram et al., 2008). Tetramer peptide containing 4 copies of peptide *ffvIk* (composed of D-amino acids and has the reverse sequence of A 16–20 peptide). Lysine was added to the C terminals and β alanine was added to provide turns. The peptides were purified by reverse-phase HPLC and analyzed by MALDI-TOF to confirm structure.

Detox gel

25 mg MAL-PEG-NHS (5000 mol wt) was dissolved in 2.5ml of 20mM phosphate buffer, pH 7.4. To this, add 7.5 mg of tetramer peptide. Nitrogen was bubbled through all solutions to remove oxygen. The solution was mixed at room temperature for 2 hrs and dialyzed with a 20kd cutoff dialysis membrane. We injected 100ul/mouse/injection. Therefore, each injection contained 300ug of the tetramer peptide.

Animals

The detox gels were administered to heterozygous APPSWE amyloid precursor protein (APPSWE-Tg2546) mouse model (Hsiao et al., 1996). These mice show rapid increases in A β levels at approximately 6 months of age with A β deposition developing in the following months although extensive amyloid burden is usually not observed until the animals are well into their second year (Kawarabayashi et al., 2001). Mice were 20–24 weeks old when experiment was started. The animals were maintained on a 12-h light: 12-h dark cycle, and animal care was in accordance with institutional guidelines in AAALAC approved facilities at Taconic Farms, NY. The mice received subcutaneous injections (100 μ L) of detox gel. One injection per month was given the first 3 months followed by 2 biweekly injections thereafter (Table 1). The mice went through the Y maze behavioural test before drug administration and prior to euthanasia. A group of mice received no injections and served as control mice group. Mice were anaesthetized with sodium pentobarbital (150 mg / kg, i.p.), perfused transaortically with phosphate buffer, and the brains processed as described previously (Kawarabayashi et al., 2001). The right hemisphere was immersion-fixed in paraformaldehyde, whereas the left hemisphere was snap-frozen for measurements of A β levels.

Tissue homogenization and sandwich ELISA assay for A β levels

Extraction and measurement of A β from brain tissue was performed as described (Kawarabayashi et al., 2001). Briefly, frozen hemi brains were homogenized followed by sonication in a 10 fold volume of Tris-buffered saline (TBS- 20 mM Tris and 137 mM NaCl, pH 7.6) containing 2% SDS and protease inhibitors (complete protease inhibitor cocktail, Boehringer Mannheim, Mannheim, Germany) followed by centrifugation at 100,000 X g for 1 hr at 4°C. The supernatant was then removed, and the pellet was sonicated with 70% formic acid (FA) and centrifuged at 100,000 X g for 20 min at 20°C. Formic acid extracts

(supernatants) were neutralized by diluting (1:20) into 1 M Tris phosphate buffer, pH 11. The supernatants were vacuum-dried and solubilized in TBS. Extracts of brain were coated on 96-well micro titer plates (MaxiSorb; Nunc, Roskilde, Denmark) in triplicates (5µl/well). The wells were blocked with 3% gelatine (1 hour at 37°C) following which the biotinylated monoclonal β -amyloid antibody 12F4 (Covance, WI that recognises only the A β -42 peptide) was added at a 1: 1000 dilution and plates incubated for 1 hour at 37°C. The wells were then washed and probed with streptavidin-horseradish peroxidase conjugate (Amersham Biosciences, at 1:10,00 dilution), for one hour at 37°C. Color was developed and read at 620 m by TMB substrate (KPL, Gaithersburg, MD) as per manufacturer's instructions. Standard curves of A β -42 (AnaSpec, CA) scaling from 6–400 mols were used.

Measurement of A β -42 in serum by ELISA

In order to examine the validity of our sink hypothesis, we measured the levels of A β -42 in the sera obtained from these mice at the end of the experiment. This was accomplished by an ELISA using immobilized sera (at 1:10 dilution). After blocking, serum A β -42 was probed with a biotinylated monoclonal antibody 12F4 (at 1: 1000 dilution, Covance, WI). ELISA was performed as mentioned in the earlier section. Standard curves of A β -42 (AnaSpec, CA) were used.

Immunohistochemistry

Serial sections (5 µm) were cut sagittally through the mouse hemisphere embedded in paraffin with rotary microtome. Every 3rd section of each series of sections will be mounted on Superfrost Plus slide. All sections were processed for amyloid beta-immunohistochemistry using a monoclonal mouse anti-human beta-amyloid antibody (G48, Covance, WI). In brief, following deparaffinizing in xylene, sections were rinsed in graded ethanols and then rehydrated to distilled water. After inactivating the endogenous peroxidase activity with hydrogen peroxidase and antigen retrieval as described in the manufacturer's literature, sections were incubated in 0.01 M phosphate buffered saline (pH 7.4) containing normal blocking serum, BSA (Jackson ImmunoResearch Labs, West Grove, PA), Triton X-100 and the specific antibody (1:200) for overnight at 4°C. The immunoreaction products were then visualized with avidin-biotin complex Vectastin elite ABC kit (Vector Lab., Burlingame, CA) and 3', 3'-diaminobenzidine as a chromogen. After thorough rinses in distilled water, sections were counterstained with hematoxylin. Following dehydration in ethanol, sections were cleared in xylene, and coverslipped in Permount® (Fisher Scientific, Fair Lawn, NJ). Immunohistology services were provided by FD Neurotechnologies (Maryland) and pathology assistance was provided by Vertinary Histology Consultants (Connecticut).

Behavioural testing- Y Maze

This test is performed as described (King and Arendash, 2002). Briefly, the mice will be handled gently, holding by tail and let to rest on arm, taking care not to let mouse plunge down head first into maze. Mice were individually placed in the centre of a Y maze, with all three arms available for exploration, for a period of 8 minutes. The Y maze is a three-arm maze with equal angles between all arms. The percentage of triads in which all three arms are represented were recorded as an alternation to estimate short-term memory of the last arms entered. After the test, data was transferred from hard copy to Excel spreadsheet. An alternation is defined as a visit to all three arms without re-entry (ABC, ACB, BAC, BCA, CAB or CBA). Mice were tested before and after detox gel administration.

Antibody levels

Antibody levels were determined at 1: 10, 1: 100 and 1: 1000 dilutions of serum using ELISA as described previously (Sigurdsson et al., 2001), in which tetramer peptide was coated onto micro titer wells (100 ng per well), stored at 37°C overnight, and then blocked with buffer containing 3% gelatine in 1× Tris-buffered saline for 2 h at room temperature. Diluted sera (1:10, 1:100 and 1:1000) were added to antigen-coated wells and incubated for 2 h at room temperature. After washing with 0.1% Tween 20 in Tris-buffered saline, plates were incubated with HRP -labelled goat anti- mouse secondary antibodies (Kirkegaard and Perry, Gaithersburg, MD at 1:5000 dilution) for 2 hours at room temperature and plates were again washed with TBST. After adding HRP substrate solution (Kirkegaard and Perry, Gaithersburg, MD), absorbance was read at 620 nm. Antibody levels to A β -42 were also measured similarly where A β -42 (instead of tetramer peptide) was coated on microtiter plates (100 ng per well) and treated as above.

Statistical analysis

All results were expressed as the mean $\pm$ SD for each group. The Mann-Whitney test was used to compare two sets of data and statistical significance (p value) was calculated.

RESULTS

We have already demonstrated that effectiveness of a novel “detox gel” system in binding A β peptides in vitro (Sundaram et al., 2008). In the present study, an attempt was made to determine its efficacy in a mouse model of AD (APPSWE-Tg2546). We injected the APPSWE (Tg2546) mice with our detox gels when the mice were 6 months old over a span of 4 months (n = 9). Uninjected mice served as control (n = 9). At the end of the study, brain and serum samples were collected and analysed. Memory tests (Y maze) were performed in all the mice one week prior to the first injection and one week after the last injection. The results are presented below.

Effect of detox gel on the body weight in APPSWE (Tg2546) mice

We examined the changes of body weight in the detox gel treated mice and non-treated mice. Body weight was measured at the beginning and end of the study. There was gain in body weight in the detox gel treated mice as shown in Figure 1, (26 ± 2.5 grams before treatment to 30 ± 3.4 grams at the end of treatment with $p < 0.002$). In the untreated group however, the mice gained no weight (27.1 ± 2.3 grams at the beginning to 27.5 ± 3.1 grams at the end of the study)

Quantitative analysis of A β -42 in brain

A β in brain was extracted by methods described in experimental methods section. A β -42 levels were measured in brain extracts by an ELISA using a commercial antibody. The two-step extraction method that we employed yields total A β . The results show that there is a reduction in the level of total A β -42 by 30% in the group of mice that received the detox gel when compared to the untreated group (Figure 2) with a statistical significance ($p < 0.001$).

Measurement of A β -42 in serum by ELISA

In order to examine the validity of our sink hypothesis, we measured the levels of A β -42 in the sera obtained from these mice at the end of the experiment. This was accomplished by an ELISA using immobilized sera (at 1:10 dilution). The results indicate that there is a 50% increase in levels of circulating A β -42 in the treated group when compared to the untreated group (Figure 3). Thus the data indicate efflux of A β -42 from brain to periphery, which is a

phenomenon, observed by other studies using A β -42 sequestering agents. This observation confirms our sink hypothesis.

Immunohistochemistry of brain sections

Immunohistology was performed on brain sections and analysed. A commercial antibody specific to A β was used. The results are promising in that there is a reduction in size of plaques in the brain of detox gel treated mice when compared to uninjected control mice sections. A β immunoreactive plaque load (mean plaque size measured as μm^2 ; mean $\pm$ SD) was significantly reduced by detox gel treatment (detox gel treated, $299 \pm 107 \mu\text{m}^2$; untreated, $483 \pm 139 \mu\text{m}^2$). Comparing mean plaque sizes across treated and untreated groups reveals a decreasing trend in plaque size of treated mice. Four out of 4 animals in the untreated group have overall plaques sizes greater than the average size of $400 \mu\text{m}^2$ for the entire group compared to only 1 animal out of 5 the treated group. A sample set of pictures representing 4 largest plaques shown confirms this observation (Figure 3). To test for the possibility that the tetramer peptide crossed over the blood brain barrier (BBB), and resulting in false positive signals in IHC, we performed an ELISA using immobilized tetramer employing the same antibody that was used in IHC. The results showed that this antibody was unable to react to tetramer peptide thus suggesting the positive signal obtained in IHC was specific to intrinsic A β (Figure 6).

Behavioural test

Detox gel treated mice and control mice were assessed on the Y maze cognitive test before and after treatment. Y maze test is a qualitative measure of intact working memory in animals that tend to explore new parts of an environment instead of returning to known parts immediately (alternation). Y maze is used to assess (without food deprivation or other aversive procedures) the normal navigation behaviours of rodents and is particularly useful as an initial test of spatial memory function in mice. The Y maze test is based on the natural drive of rodents to explore novel environments. Success in this test is indicated by a high rate of alternation (spontaneous alternation) indicating that the animals can remember which arm was entered last. The results show that there is a difference between the detox gel injected mice when compared to the non-injected counterpart (Figure 4). The percentage of alteration was 47.5 % before the injections. This improved to 61.4% after detox gel regimen. These results are statistically significant. The uninjected control mice started off with a score of 62%, which decreased over the course of the study to 49%, suggesting progressive memory loss upon aging. In this context, the observed improvements in the treated mice from initial and final measurements appear to be even more pronounced.

Test for immunogenicity to the detox gels

We tested for the presence of antibodies to our peptide to determine if there was any immune response elicited. If there was an antibody response, the antibodies might have been responsible for the observed reduction of A β - 42 peptide in treated mice. Antibody levels to tetramer RI peptide measured in sera (at various dilutions) by a standardized ELISA. As can be seen in the figure 5, injection of detox gel did not induce any measurable antibody response even at the lowest dilution (1:10). This ELISA was repeated replacing the RI tetramer peptide with the A β - 42 peptide. The results showed that there were no antibodies made against the A β - 42 (data not shown).

DISCUSSION

AD is a growing problem that exacts a tremendous monetary and human toll, significantly lowering the quality of life for those affected directly and indirectly. The treatment of AD is an unmet medical need for which no palliative or curative therapies have consistently been

shown to work. We hypothesized that the detox gel consisting of a tetrameric version of the retro-inverse peptide *ffv/lk* linked to PEG will act according to the principles of the peripheral sink mechanism to irreversibly bind and retain the toxic A β from the surroundings. We have demonstrated proof of concept for this detox gel system *in vitro* (Sundaram et al., 2008). The present study is a logistic extension of our *in vitro* studies in that it attempts to test our detox gel in a well established AD mice model (Hsiao et al., 1996).

Our concept relies on retro-inverso peptides, in which all D-amino acids are used and the change in chirality is counteracted by reversing the primary sequence, contain inter-amino acid bonds that are the most closely related isosteric replacements for the original peptide bond. These modifications preserve the major structural characteristics of the peptide backbone despite changing the native structure. Consequently, retro-inverso peptides generally have increased stability as has been demonstrated for a number of peptides, including enkephalin, glutathione, substance P, gastrin, and atrial natriuretic peptide (Chorev and Goodman, 1995, Dintzis et al., 1993, Herve et al., 1997). Studies have shown that the RI peptide *ffv/lk* derived from the native sequence KLVFF binds A β with great affinity thus showing that retro inversion does not alter its binding properties. We incorporated our retro-inverse peptide into a PEG based hydrogel formulation to help prolong its half life and reduce the probability of immunogenicity. The detox gel is injected subcutaneously into the mice and attracts A β peptides thus acting like a sink.

In AD, there is a tendency for loss of body weight with progression of disease (Morgan and Gordon, 2008, Holcomb et al., 1998). In the present study, we have observed a similar phenomenon in the uninjected controls. Interestingly, our detox gel treated mice gained weight over time. This observation appears to be a favourable outcome since it indicates reversal to normal physiology.

The data obtained on levels of A β -42 from brain extracts showed a 30% reduction in detox gel treated mice. This suggests clearance of A β -42 by the sequestering ability of our detox gel. The results further indicate that there is a 50% increase in levels of circulating A β -42 in the treated group when compared to the untreated group. This observation confirms efflux of A β -42 from brain to periphery, a phenomenon observed by other studies using A β -42 sequestering agents [9] and thus confirms our sink hypothesis. Consequently, immunohistology data also confirmed this observation thus providing additional line of evidence. Additionally, overall size of brain plaques in the detox gel treated mice was smaller than those from uninjected mice. This could be attributed to the reduced amount of A β peptides in brain of treated mice.

Improvement in memory correlates in treated mice was observed as evidenced by an increase in the percentage of spontaneous alteration. As the plaque load in brain increases, the spatial memory capacity (% alteration) decreases. In detox gel treated mice, the A β -42 load decreased as seen by ELISA and immunohistochemistry and this might have contributed to the improved alteration in this group. On the other hand, control mice showed a significant decrease in their alteration behaviour, which shows further progression in these mice.

Immunogenicity is one of the major adverse effects observed in other studies using immunotherapy to treat AD. We addressed this concern by examining the immune responses against detox gel treatment. We screened the sera obtained from the treated mice for the presence of antibody to the injected tetramer peptide. The results indicate that our formulation did not elicit an immune response. Tests to detect antibodies to A β peptides also proved negative results. The observed results confirm that the detox gel treatment induce a

non-immune based sequestering of A β peptides. This is consistent with our sink hypothesis. Further, the lack of immunogenicity in response to our detox gel therapy is in direct contrast to other antibody based therapies that failed due to immune reactions (Bayer et al., 2005, Orgogozo et al., 2003).

CONCLUSION

The present study shows that treatment with detox gel in AD mice can be effective as a therapeutic method for lowering A β levels in the APPSWE mice(Tg2546). The high affinity sequestering ability combined with the lack of immunogenicity and capacity to improve memory parameters make it an interesting drug candidate for the treatment of AD. Thus our detox gel therapy might become a welcome non-immune based therapy with a future in human clinical settings.

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Figure 1. Body weight of control and AD mice before and after treatment with detox gel
Weight of mice at the beginning and at the end of the study in the detox gel treated and untreated control group. (n = 4 in control and n = 5 in detox gel treated group)

Figure 2. Total Aβ-42 levels in brain of treated and untreated AD mice

Aβ-42 content of brain from detox gel treated and untreated mice. Aβ was extracted from brains with TBS containing 2% SDS followed by 70% formic acid and quantitated as described above. Experiments were repeated and were calculated as Mean ± SD and expressed as pmols Aβ-42 /g tissue. Graded concentrations of biotinylated Aβ-42 peptide were used as calibration standards. (n = 8 in control and detox gel treated group and p < 0.001 was obtained by a Mann-Whitney test).

Figure 3. Total Aβ-42 levels in serum of treated and untreated AD mice

Aβ-42 content of serum from detox gel treated and un-treated mice. Experiments were repeated in triplicates and were calculated as Mean $\pm$ SE and expressed as pM Aβ-42. Graded concentrations of biotinylated Aβ-42 peptide were used as calibration standards. Immobilized sera (at 1:10 dilution) was plated in triplicates. After blocking, wells were probed with a biotinylated monoclonal antibody 12F4 (at 1: 1000 dilution, Covance, WI). Detection was done using a streptavidin-horseradish peroxidase conjugate (Amersham Biosciences), followed by TMB substrate (KPL, Gaithersburg, MD). Standard curves of Aβ-42 (AnaSpec, CA) scaling from 6–400 pmols was used. (n = 8 for treated and untreated group with a statistical significance of $p < 0.002$ was obtained by a Mann-Whitney test.)

Figure 4. Immunohistology of brain sections from treated and untreated AD mice

Immunohistology of APPSWE (Tg2546) mice brain sections following subcutaneous injections of RI peptide containing detox gel (treated mice), and no injection (untreated mice). Sections were processed as mentioned above. Overall, animals in treated group had smaller plaque sizes than those in untreated group (n = 4 in control and n = 5 in detox gel treated group) Bar = 20 μ m.

Figure 5. Y maze analysis of mice before and after treatment

Effect of changes in spatial memory measured as % alteration in Y maze. % alteration during an 8 min session was measured before the treatment and at the end of treatment in groups that received detox gel and in groups that did not receive any treatment. Values are expressed as mean $\pm$ SD for a $n = 4$ in control and $n = 5$ in detox gel treated group with a $p < 0.1$ in treated and $p < 0.05$ in untreated group documented by Mann-Whitney test).

Figure 6. Immunogenicity to the detox gel in AD mice

Antibody response in mice injected with detox gel and mice that did not receive any injections. ELISA plates were coated with RI tetramer (100 ng per well), stored at 37°C overnight, and then blocked with buffer containing 3% gelatin in 1× Tris-buffered saline for 2 h at room temperature. Diluted sera (1:10, 1:100 and 1:1000) were added in triplicate to antigen-coated wells and incubated for 2 h at room temperature. After washing with 0.1% Tween 20 in Tris-buffered saline, plates were incubated with HRP -labeled anti mouse secondary antibodies (Kirkegaard and Perry, Gaithersburg, MD at 1:5000 dilution) for 2 h at room temperature. After adding HRP substrate solution (Kirkegaard and Perry, Gaithersburg, MD), absorbance was read at 620 nm. Values are expressed as mean $\pm$ SD for n = 8 in control and n = 9 in detox gel treated group.

Table 1

Summary of the mouse study groups and injection schedules

Mouse Group	Type of Injection	Frequency of Injection
Group 1 (n=9)	100 μ l detox gel with RI peptide(tetramer)	1 injection on weeks 1, 5, 9, 11, and 13
Group 2 (n=9)	No Injection	None

This table summarizes the number of mice in each group and the number of detox gel injections received.