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N.B.: When citing this work, cite the original article.

Original Publication:

Chamilly Evaldsson, Ingvar Rydén and Srinivas Uppugunduri, Isomaltitol exacerbates neutrophilia but reduces eosinophilia: New insights into the Sephadex model of lung inflammation, 2011, International Archives of Allergy and Immunology, (154), 4, 286-294.

<http://dx.doi.org/10.1159/000321820>

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Postprint available at: Linköping University Electronic Press

<http://urn.kb.se/resolve?urn=urn:nbn:se:liu:diva-19097>

Isomaltitol exacerbates neutrophilia but reduces eosinophilia – new insights into the Sephadex model of lung inflammation

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Short title

Isomaltitol in the Sephadex model of lung inflammation

Keywords

isomaltitol, Sephadex, inflammation, oedema, asthma, TNF, eosinophils, neutrophils, mucociliary clearance

Abstract

Background: We have previously examined isomaltitol in an *in vitro* static adhesion assay and were interested in investigating whether the potentially anti-inflammatory effects observed there could be relevant *in vivo*. The Sephadex-induced lung inflammation model was considered a suitable model due to the significant changes in global inflammatory endpoints seen upon provocation with Sephadex.

Methods: Male Sprague-Dawley rats were instilled intratracheally with Sephadex (5 mg/ml), vehicle (0.9% NaCl), isomaltitol (50 mg/ml) or a combination of isomaltitol and Sephadex. After 24 hours, the lungs were weighed to measure oedema and preserved for histology. Bronchoalveolar lavage fluid was used for analysis of tumour necrosis factor, cysteinyl leukotrienes, and differential and total leukocyte counts.

Results: Differential counts showed that isomaltitol increased the neutrophil component while decreasing the eosinophilia, thus asserting a modulatory role on the usually eosinophil-dominated Sephadex-induced cell profile. Isomaltitol alone also increased oedema and cysteinyl leukotrienes, and generally aggravated total inflammation in combination with Sephadex. The mechanisms were not investigated in this study, but effects could relate to a combination of isomaltitol's osmotic and structure-specific properties.

Conclusion: Our results show that isomaltitol can modulate the inflammatory response induced by Sephadex instillation in addition to have pro-inflammatory effects on its own, and may therefore provide new insights into the mechanisms of this widely used animal model. Sugar alcohols similar to isomaltitol have already been used to aid mucus clearance in cystic fibrosis patients, and it is possible that isomaltitol could also be used for this purpose.

Introduction

The Sephadex model has been used in parallel with allergen-induced lung inflammation models by many groups the last decades in order to simulate different aspects of inflammatory lung disorders in animal settings. Although the Sephadex model can be classified as an acute inflammation model, it is widely reported to show similarity in inflammatory profile to clinical asthma in several respects, e.g. inflammatory cell infiltrates dominated by eosinophils, oedema formation, local release of tumour necrosis factor (TNF), eosinophil peroxidase (EPO), eotaxin and cysteinyl leukotrienes (cysLT). Strain-related airway hyper-reactivity in response to Sephadex has also been reported [1]. These similarities explain the frequent use of Sephadex in screening models for anti-asthma drugs such as glucocorticosteroids. Another benefit of the model is the short time frame of experiments. An eosinophil-dominated lung inflammation in rats, with the traits described above, typically arises and peaks within 24 h of a single intratracheal Sephadex instillation without the use of adjuvant or prior sensitization to allergens.

We have previously described isomaltitol in an *in vitro* model of static adhesion between polymorphonuclear cells and TNF-stimulated endothelial cells [2]. In that setting, isomaltitol reduced neutrophil adhesion with roughly 40 % at concentrations between 1 mM and 1 nM. It was speculated that isomaltitol was able to interfere with adhesion molecule interactions, and that this might result in anti-inflammatory effects if used *in vivo*. In this study, we have therefore investigated the effects of isomaltitol on the lung inflammation in rats caused by a single intratracheal Sephadex instillation. The main variables measured in the current setting are oedema, cellular infiltration and inflammatory cell profile in lavage fluid, all of which are affected by isomaltitol.

The model is widely used in academic and industrial settings. Still, the mechanism behind Sephadex-induced lung inflammation is largely unclear, although natural antibodies against several polysaccharides have been shown to be present in rats [3] and are believed to contribute to the inflammation. Sephadex consists of dextran polymer beads, which do not readily change shape or disintegrate in the tissue [4]. Studies with ultrasonicated Sephadex show that the bead size matters, as fragmentation of the beads will lead to a lesser inflammatory reaction with a transient neutrophil infiltration, without accompanying eosinophilia or granulomatosis [5, 6]. On the other hand, the number of beads, but not the bead size, was found to correlate with the intensity of airway eosinophilia and neutrophilia [7]. The bead size alone does not explain the reaction, however, as similar-sized foreign-body implantations of other materials, e.g. latex [8], generally do not lead to such a pronounced eosinophilia. Administration route, number of administrations and average dosing also affects the inflammatory output obtained, as does the age of animals receiving Sephadex [9].

Based on the results from our *in vitro* study, we hypothesized findings in line with other substances that had reduced adhesion to a similar extent [10, 11]. Contrary to what we expected, isomaltitol increased the presence of neutrophils in the lung, while reducing the eosinophil infiltration caused by Sephadex. Although the underlying mechanism has not been examined, we believe that carbo-hydrate properties common to isomaltitol and dextran (Figure 1), of which Sephadex is made, may account for the effects observed. Seen in this light, our results correlate well with studies of patients with mucociliary dysfunction [12], give interesting clues to the mechanism behind the Sephadex model, and provide means to alter the inflammatory cell profiles of the model. The neutrophil-inducing property of isomaltitol also suggests new ways to boost immune responses in clinical settings.

Materials and Methods

The study design, materials and methods follow the procedures described in Evaldsson et al. 2007 [11] and 2009 [10], where more details can be found. In brief, rats were euthanized 24 hours post intratracheal installation of Sephadex. Lungs were excised for lavage, histology and visual assessment. Bronchoalveolar lavage fluid (BALF) was used for cell count, differential counts and TNF and leukotriene LTC₄, LTD₄, LTE₄ ELISA analysis. In addition to these

variables, tissue appearance, oedema formation, blood smears and thymus weight were also studied.

Figure 1 The structures of Sephadex and isomaltitol. Sephadex consists of crosslinked dextran polymers, while isomaltitol is a sugar alcohol.

Animals

Male Sprague-Dawley rats (175-200 g, 5-7 weeks of age) purchased from Scanbur BK AB (Sollentuna, Sweden), were housed at the Linköping University animal department and acclimatised for at least a week before inclusion. Food and water were supplied *ad libitum*. The Swedish National Board for Laboratory Animals' (CFN) guidelines were followed and experimental procedures were approved by the local ethics committee in Linköping (D.no. 25-04).

Lung inflammation model

A Sephadex (G-200 superfine, Pharmacia) suspension was prepared with sterile 0.9% NaCl 12-24 hours before instillation. For co-administration with Sephadex, isomaltitol was diluted

separately at twice the final concentration and mixed with an equal part of Sephadex of double concentration just prior to instillation. Where isomaltitol was the single substance to be administered, isomaltitol was diluted to 50 mg/ml in 0.9% NaCl.

Before experiment onset day one, rats were weighed. After a few minutes of anaesthesia inhalation by Isoflurane (Forene, Omnia Mutantur), animals were intratracheally instilled with 1 ml/kg of saline, Sephadex (5 mg/ml), isomaltitol (50 mg/ml, Sigma-Aldrich Chem. Co., St. Louis, MI) or a combination of Sephadex and isomaltitol. Animals were supervised until fully awake and active.

24 hours after instillation, rats were weighed again and euthanized with 250 mg/kg of sodium pentobarbital. Blood smears were made using whole blood. Lungs and thymi were excised, rinsed and weighed separately. A bronchoalveolar lavage was performed with 5 ml of NaCl/Xylocain (3.33 mg Xylocain® (AstraZeneca, Sweden)/ml 0.9% NaCl solution) instilled for 60 s before fluid withdrawal using a syringe. In later experiments, lavages were performed using the same amount of phosphate-buffered saline (PBS) instead, with comparable results. BALF was kept on ice until further use. Lungs were preserved in formalin.

BALF was centrifuged at 400 g and 4 °C for 5 min before total leukocyte counts and cell smears for differential counts were made. Since cells from Sephadex-treated rats appeared more fragile (as also noted by Cook, 1990 [13]), the Cytospin® method was preferred above a manual smearing technique. Blood and lavage cell smears were fixed in methanol.

Cytospin smears were stained in May-Grünwald solution (Merck, Sweden) for one min, rinsed in water and then dyed in Giemsa stain (Merck, Sweden) for about 25 min.

Excised formalin-fixed lung tissue was sent to the Swedish National Veterinary Institute in Uppsala for analysis. Histological sections were stained with haematoxylin-eosin, Giemsa or W. Starry, the latter for detection of potential micro-organisms.

BALF samples were analysed in triplicates in a TNF ELISA procedure, following the manufacturer's protocol (Quantikine rat TNF immunoassay, R&D Systems, Minneapolis). LTC₄, LTD₄ and LTE₄ were detected collectively (also in triplicates) with a competitive ELISA procedure, following the manufacturer's instructions (Biotrak Assay, Amersham/GE Healthcare, Uppsala, Sweden).

Statistical analysis

Groups treated with isomaltitol were compared one by one against the Sephadex or NaCl control groups, respectively, using the non-parametric Mann-Whitney U test. The exact significance was calculated with the SPSS Exact Tests module (SPSS 15.0, SPSS Inc., Chicago) and the two-tailed p value is given, as changes in both directions are of interest. Figures show box plots where the median is represented by a line. The box is limited by the first and third quartiles, thus shows the interquartile range (*IQR*). Whiskers indicate the highest and lowest observations that are not considered outliers. Any data point more than 1.5-*IQR* away from the first or third quartiles are considered an outlier (O). If the distance is more than 3(*IQR*), the data point is considered extreme (*).

Results

Systemic observations

Differential leukocyte counts in blood smears did not differ between the groups, indicating that the inflammation was largely localised to the lungs. The distribution of cells was also in the normal range, with 0-1% eosinophils, 1-3% monocytic cells, 9-14% neutrophils and 82-88% lymphocytes in blood.

Thymus weights were also compared, as it is known that some drugs, especially of the glucocorticoid group, can induce T cell apoptosis. When animals were treated with both Sephadex and isomaltitol, a decrease in thymus weight of about 10% was observed ($p = 0.036$, data not shown) compared to the NaCl group.

Treatment with Sephadex caused a slight decrease in body weight gain compared to the NaCl group ($p = 0.024$, data not shown), while the addition of isomaltitol led to a net reduction in body weight corresponding to a few per cents' loss compared to day one ($p = 0.014$).

Oedema formation aggravated by isomaltitol

All groups had readily visible signs of lung oedema and inflammation macroscopically when compared to the NaCl control group. Lungs from the vehicle control group had a smooth, light pink surface, while the Sephadex- and co-treated lungs were darker in colour, notably swollen and displayed multiple inflammatory plaques and small spotty bleedings on the surface. Lungs treated with isomaltitol alone did not show the spotty bleedings but were also swollen and slightly darker. Instillation with isomaltitol led to an increase in lung wet weight $p < 0.001$ compared to the NaCl group (Figure 2). When administering Sephadex, the median lung wet weight increased from 1.23 g (in the NaCl group) to 1.65 g. The fluid influx caused by Sephadex thus corresponded to a 30-40% increase in lung wet weight. Co-treatment with isomaltitol and Sephadex seemed to aggravate the oedema even further (median weight 1.78 g), but this could not be confirmed statistically.

Figure 2 Lung weight changes in response to Sephadex and isomaltitol. Lungs were excised 24 h after instillation and cleared of all tissues surrounding the lung lobes and trachea before weighing (wet weights). All statistical comparisons are made with the non-parametric Mann-Whitney U test. Iso50 = isomaltitol (50 mg/ml), SDX = Sephadex (5 mg/kg). The lines within the box plots denote median values.

Histopathology

Microscopy was performed to identify the cells of the inflammatory plaques and examine the tissue distribution of different cell types.

Small increases in inflammatory cells were observed in some lung sections from the vehicle control group. These increases most likely reflect minor damages from the instillation procedure, as the inflammatory cells were located to the trachea.

In sections from the Sephadex group, beads were seen interspersed throughout the lungs, with a concentration to alveoli and adjacent tissue, as could be expected according to the administration route. Inflammatory cells were mainly located to granulomas which had formed around the beads, and their adjacent tissue. The granulomas were rich in macrophages, monocytes and eosinophils with the addition of multinucleated giant cells (section A, Figure 3).

Figure 3 Haematoxylin-stained lung sections from Sprague-Dawley rats. Animals had been intratracheally instilled with **A** Sephadex (SDX, 5 mg/kg), **B** SDX (5 mg/kg) and isomaltitol (25 mg/kg), **C** NaCl (vehicle control) and **D** Isomaltitol only (50 mg/kg). Sections A and B show the typical granulomas that were seen associated with partially degraded Sephadex beads, indicated with arrows marked SDX. In addition to macrophages, monocytes, eosinophils and occasional lymphocytes, lesions in isomaltitol-treated animals had higher numbers of neutrophils both in tissue and bronchoalveolar lavage fluid.

Isomaltitol alone (section D, Figure 3) induced a more generalized pulmonary inflammation than Sephadex, though most obvious in the perivascular spaces and alveoli. The perivascular space was infiltrated with eosinophils, neutrophils, macrophages and monocytes. Alveolar septae and lumina contained occasional necrotic cells and scattered eosinophils, neutrophils and macrophages.

In animals where Sephadex and isomaltitol had been co-administrated (section B, Figure 3), a marked inflammatory reaction was observed in alveoli containing Sephadex beads, but the granulomas consisted predominantly of macrophages and monocytes, with low numbers of eosinophils and lymphocytes. The lung tissue also showed multiple foci of pulmonary consolidation. As was noted in sections from the isomaltitol control group, inflammatory cells were more homogenously spread throughout the tissue than in the Sephadex group.

Bacteria were not detected in any section, which confirms Sephadex and/or isomaltitol instillation as the cause of inflammatory changes.

Leukocyte profile

Observations of cell infiltration to lung tissue were in accordance with total leukocyte counts (TLCs) in the BALF samples. As expected from other studies, Sephadex led to increased infiltration of leukocytes compared to vehicle ($p < 0.001$). The median value changed from 0.10×10^6 in the NaCl group, to 0.31×10^6 per ml BALF in the Sephadex control group. Sephadex instillation thus nearly tripled the TLC compared to vehicle. Somewhat more surprisingly, co-administration with Sephadex and isomaltitol did not increase the TLC further, even though isomaltitol alone increased the TLC to the same levels as Sephadex (Figure 4).

Figure 4 Total leukocyte counts in broncho-alveolar lavage fluid. The lavage was performed in excised lungs with 5 ml of PBS or a mixture of saline and xylocain. All comparisons are made using the non-parametric Mann-Whitney U test. Median values are shown by a line within the box, O represents outliers. Iso50 = isomaltitol (50 mg/kg), SDX = Sephadex (5 mg/kg).

This was explained by analysing the BALF differential counts. While the Sephadex-induced TLC increase consisted of massive eosinophil and, secondarily, neutrophil influx, isomaltitol administration actually reduced the Sephadex-induced eosinophilia and resident/Sephadex-induced monocytic cell numbers while triggering a substantial neutrophil and lymphocyte influx (Figure 5). Eosinophils were nearly absent in the vehicle and isomaltitol control groups, and must therefore have been attracted by Sephadex instillation.

Figure 5 Differential cell counts in bronchoalveolar lavage fluid. The counts were made on May-Grünwald-stained Cytospin smears. All comparisons are calculated with the non-parametric Mann-Whitney U test. Iso50 = isomaltitol (50 mg/kg), SDX = Sephadex (5 mg/kg). The line within the box shows the median value, O represents outlier, * represents extreme value.

TNF

NaCl control animals expressed negligible amounts of TNF protein in their BAL fluid. Isomaltitol alone caused a very slight, but significant, increase in comparison with NaCl ($p = 0.004$) while Sephadex instillation led to a significant increase of larger magnitude ($p < 0.001$). The Sephadex group median was 25 pg/ml BALF with a corresponding range of 142 pg/ml, which shows that the Sephadex effect on TNF production varies considerably. Isomaltitol did not appear to affect the Sephadex-induced TNF levels (Figure 6).

Figure 6 TNF detected in bronchoalveolar lavage fluid by ELISA. Statistical comparisons were made with the non-parametric Mann-Whitney U test. The line within the box shows the median, O represents outlier, * represents extreme value.

LTC₄, LTD₄ and LTE₄

LTC₄, LTD₄ and LTE₄ were measured collectively in an ELISA using an antibody binding to all three leukotrienes. Sephadex instillation led to a notable increase in BALF LTC₄, LTD₄ and LTE₄ compared to the vehicle control ($p < 0.001$). The Sephadex group median value was 402 pg/ml BALF. Isomaltitol alone increased cysLT expression even further, differing significantly from both the NaCl and Sephadex controls ($p < 0.001$ and $p = 0.002$, respectively). This increase did not seem to be synergistic with the purely Sephadex-induced cysLT increase, as the leukotriene concentration did not differ significantly between the Sephadex control and the co-treated group (see Figure 7).

Discussion

Isomaltitol is one of the sugar alcohols obtained in the production of isomalt, the equimolar mixture of isomaltitol and 6-O- α -D-glucopyranosyl-D-mannitol which is used as an artificial sweetener. The carbohydrates of the mixture are resistant to enzymatic degradation by both the human dental micro-organisms and gastrointestinal flora, which makes isomalt less cariogenic and calorigenic than sucrose [14-16].

Several carbohydrate-derived structures are known to interact with cellular adhesion molecules such as selectins. Isomaltitol was therefore included in a screening model of static

adhesion between isolated granulocytes and cultivated human umbilical cord endothelial vein cells. Results from this previous study [2], showed that the presence of isomaltitol at concentrations between 1 mM to 1 nM reduced the number of adhering neutrophils to TNF-stimulated endothelial cells. It was speculated that isomaltitol might interact with adhesion molecules. Since transmigration across the endothelial layer cannot take place under the static *in vitro* conditions of our experiments, the adhesion molecules responsible for initial rolling and cell arrest are the ones most likely to account for neutrophil adhesion. E-selectin, in particular, is important for neutrophil rolling and is known to bind to some carbohydrate structures [17].

Figure 7 Cysteinyl leukotrienes C_4 , D_4 and E_4 as detected by competitive ELISA in bronchoalveolar lavage fluid. All groups are compared using the non-parametric Mann-Whitney U test. The line within the box shows the median, O represents outlier.

Inhibition of adhesion has long been suggested as a therapeutic strategy in (chronic) inflammatory disease [18]. Uridine and 4-thiouridine, although being completely different structures, showed reductive effects on static granulocyte adhesion similar to those of isomaltitol. Both uridine and 4-thiouridine have already been shown to reduce several inflammatory parameters *in vivo* [10, 11]. We therefore deemed it plausible that isomaltitol, regardless of mechanism, would give similar results.

Contrary to what we first expected, isomaltitol had an ambiguous effect on inflammation. For example, isomaltitol by itself caused an increase in lung wet weight and leukocyte infiltration, the latter almost at par with Sephadex when looking at the total leukocyte count. A closer look at the cell profiles revealed that the attracted cell types differed by treatment. Isomaltitol attracted neutrophils to a much greater extent than vehicle or Sephadex, while suppressing or reducing monocytic and eosinophil counts. The reduction in eosinophil counts was in fact

similar to those of uridine and 4-thiouridine [10, 11]. Monocytic/macrophage cells were the only cell types found in significant numbers in the NaCl control group. As this reflects the expected situation in an unprovoked (or slightly provoked, as by the instillation procedure) lung, isomaltitol's ability to reduce this number is all the more noteworthy ($p < 0.001$). Further, as active macrophages are an important source of TNF this reduction could explain the lack of a further reduction of TNF levels in spite of the apparent inflammation that is induced when animals are treated with isomaltitol.

In contrast, cysLTs, which are produced by several cell types including neutrophils, basophils, mast cells and eosinophils [19, 20], increased in response to isomaltitol. In inactive neutrophils, 5-lipoxygenase is located to the cytosol. Upon activation, the enzyme will relocate to the nuclear envelope where it will catalyze the production of LTA_4 , predecessor of LTB_4 and cysLTs. Activated neutrophils could therefore be a potential source of the cysLTs detected in BALF.

As mentioned in the introduction, the mechanism behind Sephadex-induced lung inflammation is largely unknown. The reaction is often vaguely contributed to the animals in question having an "endogenous sensitivity", assumingly to be interpreted as a cross-reaction of antibodies preformed against an antigen similar to Sephadex or dextran. However, to our knowledge such antibodies, (IgE or any other isotype), have not been detected before exposure to Sephadex. A study by Blain et al. [21], indicated that complement activation may play a role in the Sephadex reaction. Other mechanistic hints can be derived from papers where different kinds of parasite or foreign-body reactions are investigated. Like Sephadex, eggs from the parasite *Schistosoma mansoni*, will induce lung eosinophilia. The egg lesions are however T-cell mediated, more active and destructive and will have eosinophils directly associated with the eggs [22, 23]. Studies with similarly sized beads of other materials than dextran confirm that bead size alone is not the only trigger of the eosinophilic response. For example, latex beads will produce smaller lesions almost exclusively dominated by macrophages [8].

Previous studies have suggested that though there seems to be no correlation between the size of the Sephadex beads and the intensity of inflammatory cell infiltration, there does seem to be a correlation between the number of beads and neutrophilia [7]. Bjermer et al. [5] have also reported that ultrasonicated Sephadex only produce a transient neutrophil alveolitis without the accompanying eosinophilia or granulomatous reactions normally observed.

Initially, we speculated that isomalt and dextran possess structural similarities which could account for the exacerbation of the inflammatory reaction observed with isomalt. Both isomaltitol and Sephadex are carbohydrate structures, although the dextran polymer is gigantic in comparison to isomaltitol. However, the three-dimensional structure of isomaltitol in solution would be very different from that of Sephadex, making any hypothetical cross reaction between antibodies or triggering of similar receptors unlikely. On the other hand, both carbohydrates have a high capacity of affecting hydrostatic pressure. It could therefore be speculated that the osmotic properties, rather than any structure-specific traits of isomaltitol and Sephadex, affect the nature of the inflammation. In our experiments, the administered dose of isomaltitol would probably yield a solute concentration *in situ* highly capable of attracting water from the surrounding tissue. To speculate further, a massive movement of water across cell and tissue barriers would be likely to destabilize membranes and possibly even injure cells to a degree where inflammation is initiated. In order to establish whether it is an entirely osmotic effect we have observed, similar experiments with for example isomaltose would be of great interest.

In contrast to this scenario, there are conditions where a slight increase of water in the airways could be beneficial. Examples include several diseases where inefficient clearance of mucus is a problem, e.g. cystic fibrosis, chronic obstructive pulmonary disorder and severe asthma. Interestingly, inhalation of a mannitol, another small sugar alcohol, has been shown to increase the clearance rate of mucus in cystic fibrosis patients [24], and it is plausible that low doses of isomaltitol may have similar effects.

In conclusion, isomaltitol at a high dose is capable of causing airway neutrophilia and oedema, in addition to modulating the usually eosinophil-dominated Sephadex-induced inflammation model. The immuno-stimulatory effects could potentially be of interest in patients whose immune systems are compromised. Comparing the results from this study with studies of the model made by others possibly hint at a role for osmosis behind Sephadex-induced lung inflammation. The mechanisms through which isomaltitol acts are unknown, but probably involve a combination of structure specific traits and osmotic effects. Further studies with other carbohydrates or sugar alcohols of similar size and at different doses would likely provide the answer to this question.

Acknowledgements

The authors wish to express their gratitude towards Gun Hilén who has performed the leukocyte differentiation, Ricardo Feinstein at the Swedish National Veterinary Institute who has analysed the lung sections, and the Medical Research Council of Southeast Sweden for providing the necessary funding. We are also very grateful to professors Anders Rosén and Peter Pålsson (Linköping University, Sweden) for helpful comments and discussions during the preparation of this manuscript.

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