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Natramune and PureWay-C reduce xenobiotic-induced human T-cell α5β1 integrin-mediated adhesion to fibronectin

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A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Background: *In vitro* and *in vivo* studies demonstrate that nutritional supplementation reduces inflammation and inflammatory markers associated with T-cell adhesion mechanisms. Here, we investigate the effects of the nutritional supplements, Natramune (PDS-2865) and PureWay-C, on xenobiotic-induced α5β1 integrin-mediated T-cell adhesion to fibronectin.

Material/Methods: The human CD4+ lymphoblastoid cell line CEM SS was treated with combinations of bifenthrin, blocking antibodies to human β1 and α5 integrin, and nutrient supplements. After 30 minutes unattached cells were aspirated and the percent of attached cells was determined.

Results: Bifenthrin stimulated T-cell adhesion to fibronectin at concentrations between 1.0 and 100 μM with a maximal stimulation of 8.3-fold at 10 μM. At 500 μg/ml, Natramune reduced 100 μM bifenthrin-induced adhesion by nearly 90%. PureWay-C reduced by 1.5-fold the level of T-cell adhesion stimulated by bifenthrin concentrations of both 10 μM and 100 μM. The combination of Natramune and PureWay-C resulted in a 6.3 and 7.5-fold inhibition at 10 μM and 100 μM bifenthrin respectively. Antibody blocking studies demonstrated that bifenthrin induced CEM SS adhesion to fibronectin is mediated through α5β1 integrin. Inhibition of T-cell adhesion achieved by anti-integrin antibodies was further reduced with 50 and 500 μg/ml Natramune treatment. Pretreatment of fibronectin with Natramune did not alter induced T-cell adhesion to fibronectin.

Conclusions: These data demonstrate that xenobiotic-induced α5β1 integrin mediated T-cell adhesion to fibronectin is reduced by nutritional supplementation with Natramune (PDS-2865) and PureWay-C. These data suggest the possibility that inflammatory responses associated with exposure to pollutants can be mitigated by nutritional supplementation.

key words: **hemicellulose • natramune (PDS-2865) • PureWay-C • xenobiotic • inflammation • integrin**

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BACKGROUND

Natramune (PDS-2865) is a hemicellulose which contains a mixture of amino acids, oligosaccharides, glycoproteins, polyphenols and fatty acids extracted from the gramineae, poaceae, and the dioscoreaceae family of plants and mushrooms. Hemicelluloses are plant and fungal cell wall polysaccharides which are available to human metabolic enzymes. Supplementation of the diet with hemicelluloses such as Natramune (PDS-2865), arabinoxylan, arabinogalactan, and other plant and fungal cell wall polysaccharides and derivatives have been shown to have a positive impact on the function of cells of the immune system. For example, an enzymatically modified arabinoxylan from rice bran (MGN-3) has been shown to increase macrophage phagocytosis [1,2], stimulate lymphocyte cytokine production [3], and inhibit p24 production and syncytia formation in HIV-1 infected T-cells [4]. Further an extract derived from ten different herbs known as Juzen-Taiho-To (JTT) contains cell wall polysacharide components and has been shown to enhance phagocytosis, cytokine production and antibody secretion [5,6] as well as have activity against malignant glioma primarily through antiangiogenic activity [7]. Moreover, the hemicellulose nutrient mixture, Natramune (PDS-2865) has been shown to increase immune cell cytokine production, stimulate macrophage proliferation and increase natural killer cell destruction of cancer cells [8].

Inflammation is a normal and natural immunological response to infection and tissue damage. However, the inflammatory response can also be responsible for significant tissue damage. In the case of xenobiotics, the mechanisms of toxicity can converge on immune signaling systems and function and directly lead to inflammation in the absence of frank tissue damage [reviewed in 9–12]. Xenobiotic induced inflammation is an undesirable event and corrective measures have been found by guarding immune cell signaling pathways and biochemistry with nutritional supplementation [reviewed in 9]. Hemicelluloses have not been studied for xenobiotic protective properties. Indeed, vitamins, in particular Vitamin C, and other antioxidants have been the most extensively studied nutrients for the ability of supplements to protect the immune system and prevent the inflammation that results from the exposure to xenobiotics [12–18]. In this regard, a novel vitamin C formulation known as PureWay-C, has been shown to be more rapidly taken up by human T-cells when compared to other vitamin C formulations, such as Ester-C [19] and to provide the greatest protection against xenobiotic induced inflammation [12].

Here we investigate the ability of the hemicellulose nutrient mixture, Natramune, in combination with PureWay-C to reduce $\alpha5\beta1$ integrin mediated T-cell adhesion to fibronection. Adhesion to fibronectin by T-cells and other cells of the immune system has been associated with mechanisms of inflammation [20–23]. The ability of a hemicellulose to protect against the deleterious effects of a xenobiotic is a novel endeavor and while PureWay-C has been shown to be protective, until this study, the ability of vitamin C in any formulation to reduce T-cell adhesion to fibronectin has not been studied.

MATERIAL AND METHODS

Material

The pyrethroid pesticide, bifenthrin purchased from ChemServices Inc, and dissolved in DMSO at 1.0 mM and substocks were generated such that bifenthin was tested at concentrations ranging from 0.1 µM to 10 µM with final DMSO concentration at 0.1% in each test well. Lippopolysacharide was purchased from Sigma-Aldrich and was dissolved in RPMI 1640 at 1 mg/ml and added to cultures at 1 µl/ml of culture media. Natramune (PDS-2865) was proved by Innovation Laboratories, Inc., and dissolved in RPMI-1640 at 50 mg/ml mixed at 4°C overnight and vacuum filtered twice using a 0.45 µM membrane and subsequently tested at 500 µg/ml. PureWay-C was dissolved in RPMI-1640 at 5 mM and subsequently tested at 50 µM. Monoclonal antibodies to human $\beta1$ integrin, human $\alpha5$ integrin, human $\alpha4$ integrin, control goat IgG and control murine IgG_1 were purchased from R&D Systems Inc, and added to cell cultures at 50 µg/ml. Fibronectin was purchased from Sigma-Aldrich and dissolved in water at 1.0 mg/ml and 10 µg was used to coat each 16 mm diameter well of a 24 well tissue cluster plate (Fisher Scientific).

Cells and culture

The human CD4+ lymphoblastoid cell line CEM SS was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: CEM-SS from Dr. Peter L. Nara and cultured in a medium of RPMI-1640 containing 10% fetal bovine serum and 0.01% gentamycin and incubated in a water-jacketed CO_2 incubator at 37°C.

Attachment assays

CEM SS cells were collected and places in 50 ml conical tubes and centrifuged at 1000 rpm for 10 minutes. The supernatant was decanted and the cell pellet was resuspended in serum-free RPMI-1640. The cells were washed in the same way twice more to remove all serum components. Cell were counted by a heamocytometer and brought to 5×10^5 cells/ml in serum-free RPMI-1640. Next, cells were seeded at in a volume of 0.5 ml containing 2.5×10^5cells into wells of a 24 tissue cluster which had been coated by 10 µg of fibronectin and blocked by 1% gelatin. Wells were coated with fibronectin by first pipetting 0.5 ml of water into the well and then adding 10 ml of 1.0 mg/ml of fibronectin to the water. The tissue clusters containing the wells were then incubated at 37°C for one hour after which the water was removed by aspiration and 1% gelatin was then added to each well for an additional hour at 37°C. The gelatin was then removed by aspiration. For pretreatment of fibronectin with Natramune, the indicated wells were incubated with 0.5 ml of Natramune at concentrations ranging from 0.5 µg/ml to 500 µg/ml in serum-free DMEM and incubated for one hour at 37°C and then rinsed three times with serum free DMEM. Next, cells were added as described above. All additions and treatments with bifenthin, Natramune, PureWay-C and antibodies were made at the time of cell seeding. After 30 minutes the wells were aspirated to remove unattached cells and the remaining attached cells were fixed and stained with Diff Qick (Baxter Scientific). The number of cells in

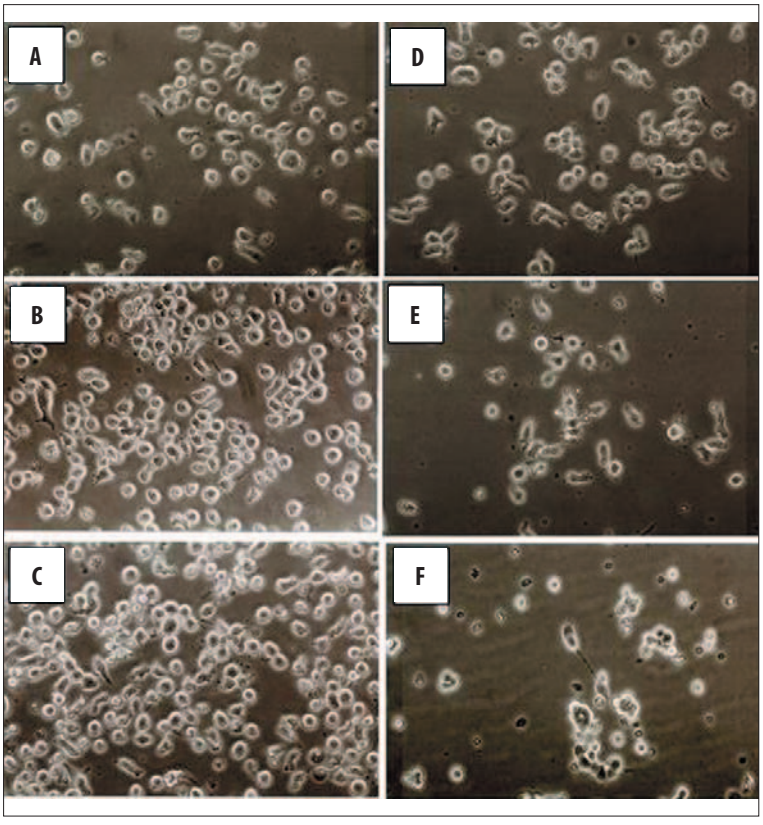

Figure 1. Photomicrograph of CEM SS T4-lymphoblastoid cells attached to a fibronectin substrate. Cells were seeded (2.5×10^5 cells/0.5 ml) in 16 mm diameter wells of a 24 well cluster which had been coated with 10 µg of fibronectin and blocked with 1% gelatin. After a 30 minute incubation, the media were removed and 0.5 ml RPMI-1640 which had been warmed to 37°C was gently added back to the wells and the cells were immediately photographed at 600 × magnification. At the time of cell seeded cell were treated with 0.1% DMSO as a solvent control (**A**); 1 µg/ml lippopolysaccharide (**B**); 10 µM bifenthrin (**C**); 10 µM bifenthrin with 50 µM PureWay-C (**D**); 10 µM bifenthrin with 500 µg/ml Natramune (**E**); and 10 µM bifenthrin with 50 µM PureWay-C and 500 µg/ml Natramune (**F**).

each of the duplicate well was determined by counting the number of cells in each of three fields/well with a 20X objective. At this magnification there are 64 fields in each well. The average number of the three fields was calculated and multiplied by 64 and this number was averaged for the two duplicate wells. This number was then divided by the total number of cells added to the well (2.5×10^5) and multiplied by 100 to give the percent of the cells attached. Each Experiment was conducted three separate times giving a total of six wells from which the number of cell attached could be determined and three measurements of the percent of cells attached. The three calculations of percent of cells attached were averaged and the standard error of the mean was determine and presented in the figures. For purposes of photomicrography, after removal of unattached cells, 0.5 ml RPMI-1640 which had been warmed to 37°C was gently added back to each well and live cells were photographed through an inverted microscope at a total magnification of 600 × using a digital camera. These cells were not then incorporated into attachment assay data.

Cell proliferation assays

CEM SS cells were seeded in T-25 flasks in 5.0 ml (at 5×10^5 cells/ml) of RPMI-1640 supplemented with 10% fetal bovine serum and 0.01% gentamycin and incubated in a water-jacketed CO_2 incubator at 37°C. At the time of seeding flasks were either untreated or treated with 500 µg/ml Natramune and incubated in a water-jacketed CO_2 incubator at 37°C. At 24 hour intervals the cells were agitated to achieve a homogeneous distribution in the medium and 10 µl was removed was then combined with 10 µl of 0.4% trypan and viewed on a hemocytometer. An area on the hemocytometer grid equivalent to 0.1 µl was counted and the number of cells excluding trypan was determined and use to determine increases in cell population per ml of media as a measure of proliferation.

Results

Bifenthrin stimulated T-cell adhesion to fibronectin (Figure 1) at concentrations ranging between 1.0 µM (10^{-6} M) and 100 µM (10^{-4} M) by over five-fold with a maximal stimulation of 8.3-fold at 10 µM (Figure 2 and Table 1) when compared to 0.1% DMSO treated cells. At 10 nM and 100 nM, bifenthrin did not significantly stimulate attachment (Figure 2 and Table 1). Bifenthrin-induced T-cell adhesion to fibronectin was significantly reduced when the cells were treated with goat polyclonal blocking antibodies to α5 and murine monoclonal antibodies to β1 integrin (Figure 3). Murine monoclonal antibodies to α4 integrin did not reduce bifenthrin-induced attachement to fibronectin. Control normal goat IgG did not affect bifenthrin-induced T-cell adhesion to fibronectin (Figure 3) as well as nonspecific murine IgG_1 (data not shown).

Lippopolysaccharide, a known stimulator of T-cell attachment to fibronectin [24], promoted a 5.7-fold increase in T-cell attachment to fibronectin (Figures 1,2 and Table 1). At 500 µg/ml, Natramune completely blocked 100 µM bifenthrin-induced adhesion to control levels and reduced the effect of 10 µM bifenthin by five-fold (Figures 1,2 and Table 1). Natramune alone did not affect T-cell proliferation (data not shown). PureWay-C also reduced the level of T-cell adhesion at 10 µM and 100 µM bifenthin by 1.5-fold at both concentrations (Figures 1,2 and Table 1). The com-

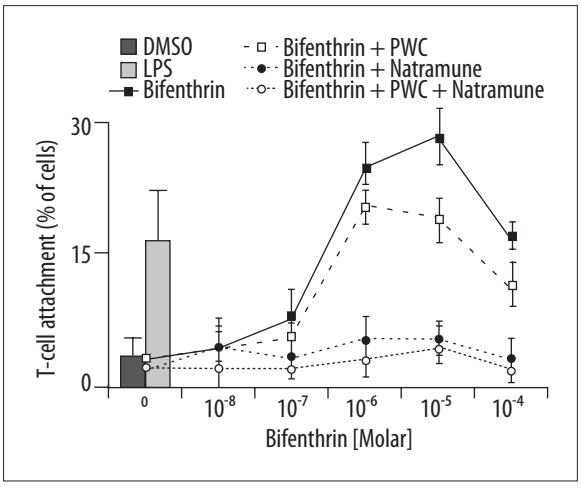

Figure 2. Natramune and PureWay-C inhibit bifenthin stimulated T-cell adhesion to fibronectin. CEM SS cells were seeded at 2.5×10^5cells per 0.5 ml in 16 mm diameter wells of a 24 well cluster which had been coated with 10 µg of fibronectin and blocked with 1% gelatin. At the time of cell seeding the indicated treatments and/or combinations of 0.1% DMSO, 1 µg/ml lippopolysaccharide (LPS), 50 µM PureWay-C (PWC) and 500 µg/ml Natramune were added to the appropriate wells. After a 30 minute incubation, the media were removed by aspiration to remove unattached cells and the attached cells were fixed and stained and counted to determine the percent of attached cells as described in the Materials and Method section. Bifenthrin was dissolved in DMSO to achieve the indicated cell treatment concentrations such that and all the various bifenthrin treatments contained 0.1% DMSO in the test wells. LPS, PWC and Natramune were all dissolved in RPMI-1640.

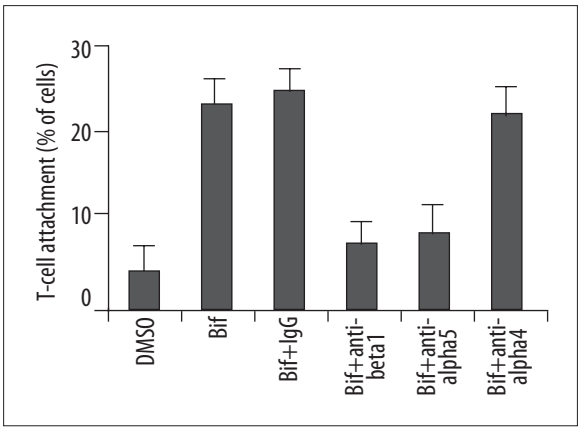

Figure 3. Bifenthin stimulates CEM SS cell attachment to fibronectin through α5β1 integrin. CEM SS cells were seeded at 2.5×10^5 cell per 0.5 ml in 16 mm diameter wells of a 24 well cluster which had been coated with 10 µg of fibronectin and blocked with 1% gelatin. At the time of cell seeding the indicated IgGs were added at 50 µg/ml along with either 0.1% DMSO or 10 µM bifenthrin (Bif) dissolved in DMSO (final 0.1% DMSO in the bifenthrin treatment wells). The antibodies used were normal goat IgG (IgG), monoclonal murine IgG to β1 integrin (anti-Beta1), goat IgG to α5 integrin (anti-alpha5) and monoclonal murine IgG to α4 integrin (anti-alpha4). Normal murine IgG_1 was also tested and had no effect on bifenthrin-mediated attachment to fibronectin (data not shown). Murine monoclonal IgG to α4 integrin had no effect and serves as a control. After a 30 minute incubation, the media were removed by aspiration to remove unattached cells and the attached cells were fixed and stained and counted to determine the percent of attached cells as described in the Materials and Method section.

Table 1. Bifenthrin-mediated T-cell attachment to fibronectin is reduced by Natramune and PureWay-C.

	Percent T-cell attachment to Fibronectin			
	None	+PWC	+Natramune	+Natramune +PWC
Treatments				
No addition	3	N.D.	N.D	N.D.
DMSO	3	3	2	2
LPS	17	12	5	4
Bifenthrin				
10^{-8} M	4	4	4	2
10^{-7} M	7	5	3	2
10^{-6} M	22	18	5	3
10^{-5} M	25	17	5	4
10^{-4} M	15	10	3	2

CEM SS cells were seeded (2.5×10^5cell per 0.5 ml) in wells of a 24 well cluster which had been coated with 10 µg of fibronectin. The indicated treatments and additions were made at the time of cell seeding. Dimethylsulfoxide (0.1%) was added as a vehicle control for bifenthin. Bifenthrin was added at the indicated concentrations without additions of 50 µM PureWay-C (PWC) (none) or with the indicated additions (+). Lippopolysaccharide (LPS) was added at 1 µg/ml as a known stimulator of adhesion to fibronectin. PureWay-C (PWC) (50 µM) and Natramune (500 µg/ml) were added separately and in combination as indicated. After 30 minutes, the cell media were removed by aspiration to remove unattached cells and the attached cells were fixed and stained and counted to determine the percent of attached cells as described in the Materials and Method section.

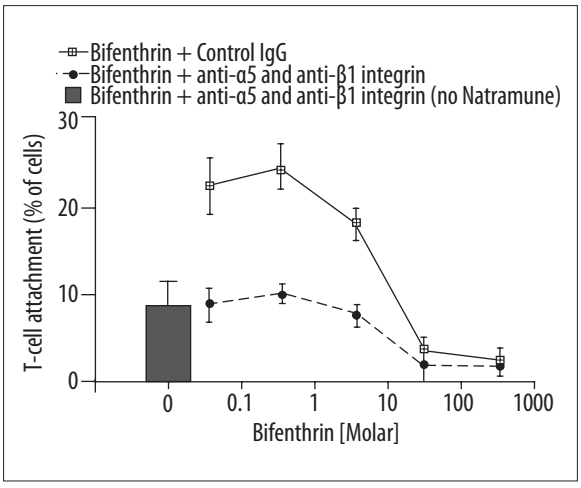

Figure 4. Natramune inhibits α5β1-mediate T-cell adhesion through non-blocking mechanisms. CEM SS cells were seeded at 2.5×10^5 cell per 0.5 ml in 16 mm diameter wells of a 24 well cluster which had been coated with 10 µg of fibronectin and blocked with 1% gelatin. At the time of seeding, all cells were treated with 10 µM bifenthrin and increasing concentrations of Natramune as indicated. Also added at the time of cell seeding was 100 µg/ml of control IgG (hatched squares) or a combination of 50 µg/ml of both anti α5 and ant-β1 integrin (closed circles). For comparison, cells treated with the anti-integrin antibody combination also received no Natramune treatment (black bar). After a 30 minute incubation, the media were removed by aspiration to remove unattached cells and the attached cells were fixed and stained and counted to determine the percent of attached cells as described in the Materials and Method section.

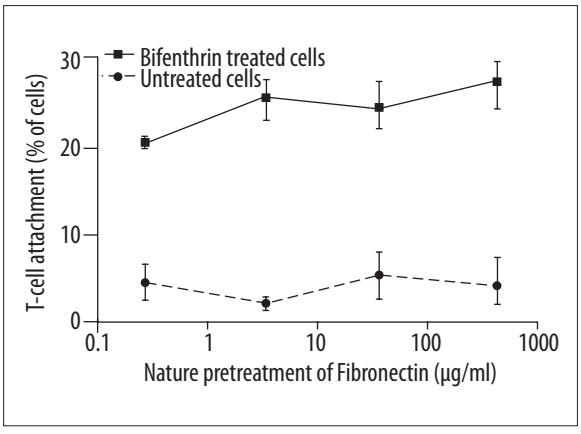

Figure 5. Pretreatment of Fibronectin with Natramune. Fibronectin adsorbed wells (10 µg/well) were treated with increasing concentrations of Natramune as indicated. CEM cells were then seeded in the wells in the presence (squares) or absence (circles) of 10 µM bifenthrin. After a 30 minute incubation, the media were removed by aspiration to remove unattached cells and the attached cells were fixed and stained and counted to determine the percent of attached cells as described in the Materials and Method section.

bination of Natramune and PureWay-C was most effective at blocking bifenthrin-induced T-cell adhesion to fibronectin with a 6.25 and 7-5-fold inhibition at 10 µM and 100 µM bifenthrin respectively (Figures 1,2 and Table 1).

To investigate if Natramune was blocking α5β1 integrin mediated adhestion to fibronectin, increasing concentrations of Natramune was added to the T-cell in the presence and absence of constant levels of bifenthrin and/or combined anti-α5 and anti-β1 integrin (Figure 4). Natramune had no effect on T-cell adhesion at concentrations lower than 50 µg/ml. At 50 and 500 µg/ml, Natramune reduced T-cell adhesion by 17% and 13% respectively (Figure 4). A combination of anti-α5 and anti-β1 integrin blocking antibodies had a maximal inhibition of 65% which was significantly less than observed by Natramune alone (Figure 4). Further, the maximum inhibition observed by the combination of blocking antibodies was further reduced by 78% with Natramune (Figure 4).

In order to determine if Natramune was blocking integrin binding sites on the fibronectin, 10 µg fibronectin was pretreated (adsorbed) with concentrations of Natramune ranging from 0.5 to 500 µg/ml (Figure 5). Natramune did not block attachment sites on fibronectin as increasing concentrations of Natramune pretreatment, bifenthrin-stimulated T-cell adhesion actually increased slightly with no significant difference between 50 and 500 µg/ml pretreatment (Figure 5).

Natramune did not stimulate T-cell proliferation over a three day period when treated and untreated equivalently seeded cell populations were measured using a hemocytometer (data not shown).

Discussion

Hemicelluloses are polysaccharide heteropolymers found in the cell wall of plants and fungi which are less complex (shorter polymer) and organized than the predominant cell wall polysaccharides such a cellulose. Consequently, hemicelluloses are easily hydrolyzed and extracted from cell walls. The sugar monomers that comprise hemicellulose include xylose, mannose, galactose, rhamnose and arabinose and, again, unlike cellulose, the glycosidic bonds between these sugars are substrates for many hemicellulase enzymes. Hemicellulose supplementation in the human diet has been found to have potent immune enhancing activity and consequently has received a great deal of attention as clinically beneficial nutraceuticals. For example, plant and fungal hemicellulose derivatives and bacterial exocelluloses have been shown to fight infectious fungal and viral diseases including HIV, boost immune system functions including antibody and cytokine production and phagocytosis, reduce tissue damage, prevent osteoporosis and provide antioxidant and free radical scavenging activity [1–4,25–29]. Many dietary fiber supplements are commercially available which contain various forms of hemicelluloses in order to help people take advantage of these dietary benefits. Natramune (PDS-2865) is one such product and has been shown to increase immune cell cytokine production, stimulate macrophage proliferation and increase natural killer cell destruction of cancer cells [8].

In addition to bolstering the immune system and providing protection from infections and other degenerative processes, dietary supplements have also been shown to provide

protection from exposure to xenobiotics [9–18,30–32]. For example, tissue damage and disease associated with exposure to environmental toxins such as cigarette smoke, pesticides, dioxins, methyl mercury, and pharmaceutical drugs have been shown to be mitigated at least in part by dietary supplementation with hemicelluloses, vitamin C, N-acetyl cysteine, inulin, and linoleic acid [9–18,30–32].

Here we have shown that Natramune (PDS-2865) reduces xenobiotic induced T-cell adhesion to fibronectin through α5β1 integrin. Our competition studies suggest that Natramune is not blocking integrin function by competing for binding sites on the integrins or the fibronectin substrate, but may rather be affecting distal changes in cell function. This is consistent with previous observations that the arabinoxylan derivative, MGN-3 increases ICAM-1 expression in human peripheral blood cells which is the β2 integrin target [4]. Further, the role of the hemicellulose, arabinoxylan in plant cells has been suggested to be stabilization of the cell cytoskeleton through interaction with cell surface transmembrane proteins [33] and this interaction has been shown to be disrupted by RGD peptides [34] which is the peptide sequence target for mammalian integrins. Further, MGN-3 has been shown to elevate macrophage nitric oxide production [2] and is a commonality to signaling pathways through integrin ligands which have also been shown to stimulate nitric oxide production and to be linked to cytoskeletal rearrangements [35]. Therefore, while the cellular mechanisms of action of Natramune and other hemicelluloses are not well studied, the limited research that does exist suggests that the beneficial effects of these cell wall derivatives on the immune may be linked to increased phagocytosis, migration and secretion, by affecting the balance between cytoskeletal rearrangement and stability.

Conclusions

A healthy diet is important for normal physiological function. Nutrients available through the diet also have importance in protecting cells of the body and normal physiological functions from the adverse effects of exposure to environmental toxins and other xenobiotics. Here we find that the hemicellulose nutrient mixture, Natramune (pds-2865), alone and when combined with PureWay-C, protects T-cells pesticide induced T-cell adhesion to fibronectin through the α5β1 integrin. T-cell adhesion to fibronectin through integrins is associated with the mechanisms of inflammation. Therefore this study suggests the possibility that dietary supplementation with Natramune (PDS-2865) and PureWay-C provides important anti-inflammatory protection from exposure to the inflammatory activity of common household pollutants such as the pyrethroid pesticides. Due to the increasing use of household chemicals, it may be important to reconsider the nutritional requirements and incorporate dietary supplements, such as Natramune (PDS-2865) and PureWay-C as a routine barrier to xenobiotic induced disease.

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