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An open-label, randomized clinical trial to assess the immunomodulatory activity of a novel oligosaccharide compound in healthy adults

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ABSTRACT

Background: Rice Bran Arabinoxylan Compound (RBAC) is a nutritional supplement produced by enzymatic hydrolysis of hemicellulose B derived from rice bran. Several *in vitro* studies and clinical reports have shown RBAC to possess promising immunomodulating effects, specifically with respect to natural killer cell and cytokine activity. The concept of a true immunomodulator is an agent possessing a broad range of activity dependent upon the existing state of health and immunity in the individual host. The present study investigated the immunomodulatory effect of RBAC in a healthy adult human population over 60 days by assessing changes in natural killer cell cytotoxicity (NKCC) and cytokines and growth factors. Subjects participated in a two-group, randomized intervention, where one group (n=10) consumed 1 gram/day and the other (n=10) consumed 3 gram/day. Safety and tolerability of RBAC were assessed with total bilirubin, total protein, creatinine, and liver function tests.

Results: We found that both groups had similar responses for NKCC, cytokines, and growth factors. The NKCC peaked at 1 week, whereas interferon-γ, tumor necrosis factor-α, interleukins-1α, -1β, -8, and -10, and epidermal growth factor peaked at 30 days. All subjects tolerated the supplement without any adverse reactions.

Conclusions: Our results showed transient, bi-directional, immune marker effects consistent with true, multifactorial immunomodulation rather than simply immunostimulation or immunosuppression. Given our findings, the immunomodulatory activity of RBAC merits study

in conditions where the immune system is functionally compromised (e.g., otherwise-healthy smokers and HIV/AIDS or cancer patients). RBAC may not only help to destroy tumor cells and viruses directly, but also increase the activity of immune cells, thereby optimizing the immune system, especially NKCC, which can increase the chance and speed of host recovery.

Keywords: Rice bran, arabinoxylan compound, activity of immune cells, immunomodulation, HIV/AIDS, and cancer

BACKGROUND

Natural Killer Cells. The understanding of natural killer (NK) cells has evolved considerably since their initial characterization in 1975 as large granular cytotoxic lymphocytes of the innate immune system. While they lack antigen-specific cell surface receptors, NK cells possess the ability to recognize and destroy transformed cells. This is accomplished via the release of granzyme and perforin granules, which act to induce cell death through apoptosis. To modulate their cytotoxic actions and maintain tolerance for normal human cells, NK cells possess a diversity of activating and inhibitory receptors.

Cells transformed by viral invasion or malignant progression can be identified and eliminated in their nascent stages by various immune effector cells [1]. Animal and human studies show that NK cells play a central role in the immuno-surveillance of transformed cells [2;3]. The majority of the NK cell population exists in a resting state. Thus, peripheral blood NK cell count provides little correlation with efficacy, and NK cell cytotoxicity (NKCC) is typically utilized to monitor levels of NK cell function [4].

NK cell functions also include the production of pro-inflammatory and immunosuppressive cytokines, including: interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), monocyte chemotactic protein-1 (MCP-1; a.k.a. CCL2), and interleukin (IL)-10. Tumor cell identification and elimination are further facilitated by the actions of IFNs and other factors [5]. Macrophage and T cell responses are also modulated via the generation of IFN-γ and IL-10 by NK cells.

Immunomodulation. An immunomodulator may be defined as a substance that modulates the response of the immune system in its protection of the host organism. In the human body, this effect may involve augmenting or reducing the activation of specialized cells, antibodies, or immunochemical (e.g., cytokines) production and other specific modifications of the body's environment and physiology.

Rice Bran Arabinoxylan Compound (RBAC) is a commercially-available medicinal glyconutrient complex. In the literature, it appears under several trade names (including MGN-3 and Biobran) with demonstrated immunomodulator activity *in vivo* in mice and humans and *in vitro* in cancer cell lines [6-8].

In vitro studies have shown that varying RBAC concentrations enhance macrophage phagocytic activity and the activities of IFN-γ, TNF-α, and IL-6 cytokines [8;9]. However, the effect of RBAC in the normal, healthy population remains largely unknown. Given the potential for human health benefit apparent in prior clinical case and biologic mechanistic studies, it is clear that thorough investigation of RBAC immunomodulator activity is warranted [10]. In this

pilot study, we investigated the effect of a 60-day course of RBAC on NKCC, cytokines, and growth factors in a sample of healthy adults. Adverse reactions were monitored, along with total bilirubin, total protein, creatinine, and liver function tests to reconfirm existing documentation of safety and tolerance in humans.

METHODS

Study Participants. Participants (n = 20) were recruited by referrals at the University of Miami Miller School of Medicine during 2010. The study was conducted with the approval of the University of Miami Institutional Review Board for human subjects research, and all participants signed informed consent and HIPAA forms before participating in the study. The sample was comprised of 40% males (n = 8) and 60% females (n = 12) with a mean age of 33.6 years (Standard Deviation = 13.2; Range = 20, 66). The racial/ethnic distributions of the subjects were as follows: 55% white, non-Hispanic (n = 11), 35% Hispanic (n = 7), and 10% Asian/Pacific Islander (n = 2).

Intervention. RBAC (Daiwa Pharmaceutical Inc., Tokyo, Japan) is a nutritional supplement that contains an active ingredient patented and manufactured exclusively since 1990. It is sold as a functional food in Japan. RBAC is an oligosaccharide complex produced via hydrolysis of rice bran hemicellulose B by *Lentinula edodes, Coriolus versicolor, and Schizophyllum commune* mycelia enzyme activity. It is spray-dried into a water-soluble powder and contains the following excipients: microcrystalline cellulose, hypromellose, sucrose fatty acid ester, and potassium acetate. The result is an oligosaccharide complex principally consisting of arabinose and β-1,4-xylopyranose moieties with lesser quantities of other polysaccharide chains, including β-glucans. Subjects participated in a two-group, randomized intervention. The dosage range recommended by the manufacturer is 1-3 gram/day; therefore one group was given 1 gram/day (n = 10) and the second group given 3 gram/day (n = 10) for 60 days. All participants completed the study without any attrition.

Assessments, Sample Collection, and Processing. Each participant completed a basic demographics and medical history questionnaire at the baseline assessment. Venous blood was obtained at 5 different time points (baseline, 48 hours, 1 week, 30 days, and 60 days). Blood samples were collected into heparin, SST, and EDTA tubes and delivered to the laboratory within 2 hours of collection. All EDTA blood specimens were subjected to complete blood cell counts and auto 5-part differential count determinations by a fully-automated Coulter AcT5 hematology analyzer (Beckman Coulter, Fullerton, CA). Flow cytometric enumeration of T, B, and NK cell subsets were performed on a 4-color flow cytometer, FACS Calibur (BD Biosciences, San Jose, CA), and the different cell populations were analyzed using Cell Quest Pro software (version 5.2, BD Biosciences, San Jose, CA).

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque gradient centrifugation. PBMC were recovered from the gradient interface and washed in phosphate buffered saline. Blood was diluted with 1:1 RPMI 1640 (Gibco, Grand Island, NY), layered over Ficoll-Hypaque solution (Pharmacia, Piscataway, NJ), and centrifuged for 30 minutes at 1,500 rpm at ambient temperature. The PBMC were collected, washed with RPMI 1640, and counted and assessed for viability in trypan blue dye. Plasma for cytokine detection was separated and

stored at -80°C till used. Total bilirubin, total protein, creatinine, alanine transaminase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) were assessed on an Olympus AU5400 chemistry auto-analyzer, which uses multi-wavelength diffraction grating spectrophotometry.

Flow Cytometry-Based NKCC Assay. The NKCC assay by flow cytometry was performed based on two previously published methods with some modifications [11;12]. K562 cells were purchased from American Type Culture Collection (Rockville, MD). 3, 39-Dioctadecyloxacarbocyanine perchlorate (DiO; Sigma, St. Louis, MO) was dissolved in dimethyl sulfoxide (Sigma, St. Louis, MO) to a concentration of 3 mM. Aliquots were frozen at -30° C and thawed for each experiment. RPMI media with and without phenol red were obtained from Gibco (Carlsbad, CA). Complete medium (CM) consisted of RPMI medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Propidium iodide (PI) (Sigma, St. Louis, Mo.) was dissolved in CM to obtain a working solution of 10 mg/100 ml. Fetal bovine serum was obtained from Cellgro (Manassas, VA).

Effector (PBMC) and target cells (10,000 cells) were added to tubes to create effector-to-target (E:T) ratios within a range from 25:1 to 6.25:1. A total of 130 μl of PI was added, and the tubes were centrifuged for 30 seconds at 1,000 *g* to pellet the effector and target cells together in the presence of the PI. These cell mixtures were incubated for 2 hours at 37° C in a 5% CO_2 incubator. Controls consisted of target cells only plus PI and effector cells only plus PI and were set-up to detect spontaneous lysis and nonviable effector cells, respectively. After the cells were incubated together, they were analyzed by flow cytometry. The forward and side-scatter parameters were adjusted to accommodate the inclusion of both target and effector cells within the acquisition gate to acquire a total of 20,000 events. Data were analyzed using CellQuest pro software (BD Biosciences, San Jose, CA). Percent lysis was calculated as: percent lysis = [Cells positive for both DiO and PI/Total DiO labeled cells] * 100. The same equation was used to calculate the percent spontaneous lysis for the sample containing only target cells. The spontaneous lysis was subtracted from the experimental values for each sample.

Multiplex Cytokine and Growth Factor Testing. Due to their central role as signaling compounds in the immune system, cytokines and growth factors are involved in a variety of immunological, inflammatory, and infectious diseases. New microarray based biochip cytokine technologies combine the latest technological advances with innovative system design to present a fully-automated system for rapid multiplex testing. It enables up to 12 cytokines and growth factors to be detected simultaneously in a single sample, providing valuable information related to each molecule being tested and possible associations between them in each sample. This system saves time, costs, and resources and also provides high-quality, reliable results.

Cytokine and growth factor levels in plasma specimens were measured using a biochip array system, Evidence Investigator™ (Randox Laboratories Ltd., Crumlin, UK) as reported previously [13]. The testing platform consists of biochips secured in the base of a well placed in a carrier holding nine biochips in a 3×3 format. Each biochip is coated with the capture antibodies specific for each of the 12 cytokines and growth factors (IL-2, IL-4, IL-6, IL-8, IL-10, IL-1α, IL-1β, IFN-γ, TNF-α, MCP-1, vascular endothelial growth factor [VEGF], and epidermal growth factor [EGF]) on a particular test region. A sandwich chemiluminescent assay was

performed with 100 µl plasma using reagents (including the calibrators and controls) and protocols supplied by the same manufacturer. The light signal generated from each of the test regions on the biochip was detected using a charge-coupled detector camera and imaging system and compared with a calibration curve generated with known standards during the same run. All specimens were run in duplicate, and the concentration of a cytokine present in each plasma specimen was calculated from the standard curve and reported in pg/ml.

Statistical Analysis. Data were analyzed using SPSS 19 (IBM Inc., Chicago, IL) for Windows. Frequency and descriptive statistics were calculated on all variables. We used repeated-measures analysis of variance (ANOVA) to examine the interaction effect of group (1 gram/day versus 3 gram/day) by time (baseline, 48 hours, 1 week, 30 days, and 60 days) for NKCC, and for cytokines, growth factors, creatinine, total bilirubin, total protein, ALT, AST, and ALP the time factor was baseline, 1 week, 30 days, and 60 days. We first examined the interaction effect of the differences between group values across time. If no interaction effect was found, we then determined if the main effects of time and group were significant for the entire sample. A statistically significant interaction or the main effect of time was further examined using simple effects pairwise t-tests. We used the ώ Huynh-Feldt correction factor to adjust the degrees of freedom for the averaged tests of significance, as the most conservative test of the repeated measures effect. The criterion for statistical significance was $\alpha = 0.05$.

RESULTS

Safety Measures. Table 1 shows creatinine, total protein, total bilirubin, ALT, AST, and ALP for both groups. No significant differences were observed for any of these measures between the two groups from baseline to 60 days. For the 1 gram/day group, no subject's creatinine value was higher than 0.3 mg/dL more than the upper limit (< 1.5 standard deviation [SD] from the mean [M]), and no value was less than the lower limit. Total protein and total bilirubin were within the reference range at all time points. All ALP values were within the reference range, except for 1 value -2 U/L at 60 days. All AST values were within the reference range, except for 1 value -4 U/L at 1 week. All ALT values were within the reference range. For the 3 gram/day group, no subject's creatinine value was higher than 0.39 mg/dL more than the upper limit (< 1.5 SD from the M), and no value was less than the lower limit. Total protein was within the reference range at all time points. No total bilirubin value was higher than 0.5 mg/dL more than the upper limit (< 2.5 SD from the M). One ALP value was +4 U/L at baseline, 1 value was +3 U/L at 30 days, and 1 value was +4 U/L at 60 days. One AST value was +5 U/L at baseline, 1 value was +4 U/L at 1 week, 1 value was +41 U/L at 30 days, and 1 value was +3 U/L at 60 days (the first value was 1 subject, and the last 3 values were 1 subject). All ALT values were within the reference range, except for 1 subject, who had values of +4, +5, +13, +26, and +17 U/L for all time points, respectively.

Table 1. Creatinine, Protein, Bilirubin, Alanine Transaminase, Aspartate Aminotransferase, and Alkaline Phosphatase at Baseline through 60 Days

Measure	Time	Total Sample (n=20)	1 Gram/Day (n=10)	3 Gram/Day (n=10)
Creatinine	Baseline	0.90±0.24 (0.59, 1.42)	0.86±0.21 (0.59, 1.23)	0.94±0.27 (0.66, 1.42)

(mg/dL)	1 Week	0.90±0.22 (0.60, 1.41)	0.86±0.17 (0.66, 1.29)	0.95±0.26 (0.60, 1.41)
	30 Days	0.90±0.22 (0.60, 1.37)	0.81±0.17 (0.63, 1.11)	1.00±0.24 (0.60, 1.37)
	60 Days	0.86±0.21 (0.62, 1.36)	0.85±0.21 (0.62, 1.36)	0.88±0.23 (0.62, 1.22)
Total Protein (g/dL)	Baseline	7.4±0.4 (6.7, 8.1)	7.4±0.4 (6.8, 8.0)	7.4±0.4 (6.7, 8.1)
	1 Week	7.6±0.5 (6.6, 8.2)	7.7±0.4 (7.2, 8.1)	7.5±0.6 (6.6, 8.2)
	30 Days	7.3±0.5 (6.7, 8.3)	7.3±0.4 (6.8, 7.9)	7.4±0.5 (6.7, 8.3)
	60 Days	7.2±0.4 (6.6, 8.0)	7.2±0.2 (6.9, 7.6)	7.1±0.5 (6.6, 8.0)
Total Bilirubin (mg/dL)	Baseline	0.61±0.28 (0.3, 1.4)	0.48±0.08 (0.3, 0.6)	0.73±0.36 (0.4, 1.4)
	1 Week	0.61±0.23 (0.4, 1.2)	0.52±0.12 (0.4, 0.8)	0.69±0.29 (0.4, 1.2)
	30 Days	0.62±0.33 (0.3, 1.6)	0.47±0.13 (0.3, 0.7)	0.76±0.41 (0.3, 1.6)
	60 Days	0.58±0.32 (0.3, 1.7)	0.53±0.17 (0.3, 0.8)	0.63±0.42 (0.3, 1.7)
Alanine Transaminase (U/L)	Baseline	17.4±8.6 (8, 44)	16.5±6.9 (8, 30)	18.2±10.3 (9, 44)
	1 Week	19.5±10.4 (8, 53)	19.1±8.3 (11, 31)	19.9±12.7 (8, 53)
	30 Days	18.3±12.6 (8, 66)	15.9±6.5 (9, 28)	20.7±16.8 (8, 66)
	60 Days	18.4±12.2 (7, 57)	17.3±9.0 (8, 37)	19.5±15.2 (7, 57)
Aspartate Aminotransferase (U/L)	Baseline	20.9±6.0 (12, 35)	20.7±5.5 (12, 28)	21.1±6.6 (16, 35)
	1 Week	21.7±7.8 (6, 38)	21.1±8.9 (6, 38)	22.3±7.0 (15, 34)
	30 Days	23.6±12.2 (13, 71)	19.9±5.1 (13, 28)	27.2±16.1 (15, 71)
	60 Days	19.7±5.9 (10, 33)	19.6±5.7 (10, 30)	19.7±5.7 (10, 30)
Alkaline Phosphatase (U/L)	Baseline	65.6±22.4 (38, 119)	61.8±21.1 (38, 93)	69.4±24.1 (38, 119)
	1 Week	67.4±21.7 (36, 113)	64.3±19.7 (41, 94)	70.4±24.1 (36, 113)
	30 Days	67.5±22.2 (37, 118)	63.6±17.0 (37, 89)	71.4±26.7 (41, 118)
	60 Days	65.3±22.5 (31, 119)	60.8±18.8 (31, 85)	69.8±25.8 (39, 119)

NOTE: Values are mean ± standard deviation (minimum, maximum).

NKCC. Figure 1 shows the changes in NKCC for both groups from baseline to 60 days. We found a significant effect for time ($F[3.0,54.0] = 7.2$, $p = 0.001$), but the group by time interaction ($p = 0.85$) and the main effect of group ($p = 0.48$) were non-significant. The ώ degrees of freedom Huynh-Feldt correction factor for the within-subjects effects was 1.000. For the total sample, NKCC peaked at 1 week (M = 30.24; standard error [SE] = 2.07, 95% confidence interval [CI]: 25.88, 34.56) and was significantly higher than the baseline (M = 22.38; SE = 2.31, 95% CI: 17.52, 27.24; $p = 0.016$), 48 hour (M = 23.86; SE = 2.13, 95% CI:

19.38, 28.34; p = 0.028), 30 day (M = 20.32; SE = 1.71, 95% CI: 16.73, 23.91; p = 0.001), and 60 day (M = 20.32; SE = 2.51, 95% CI: 15.05, 25.59; p = 0.001) values. No other values were significantly different among the others.

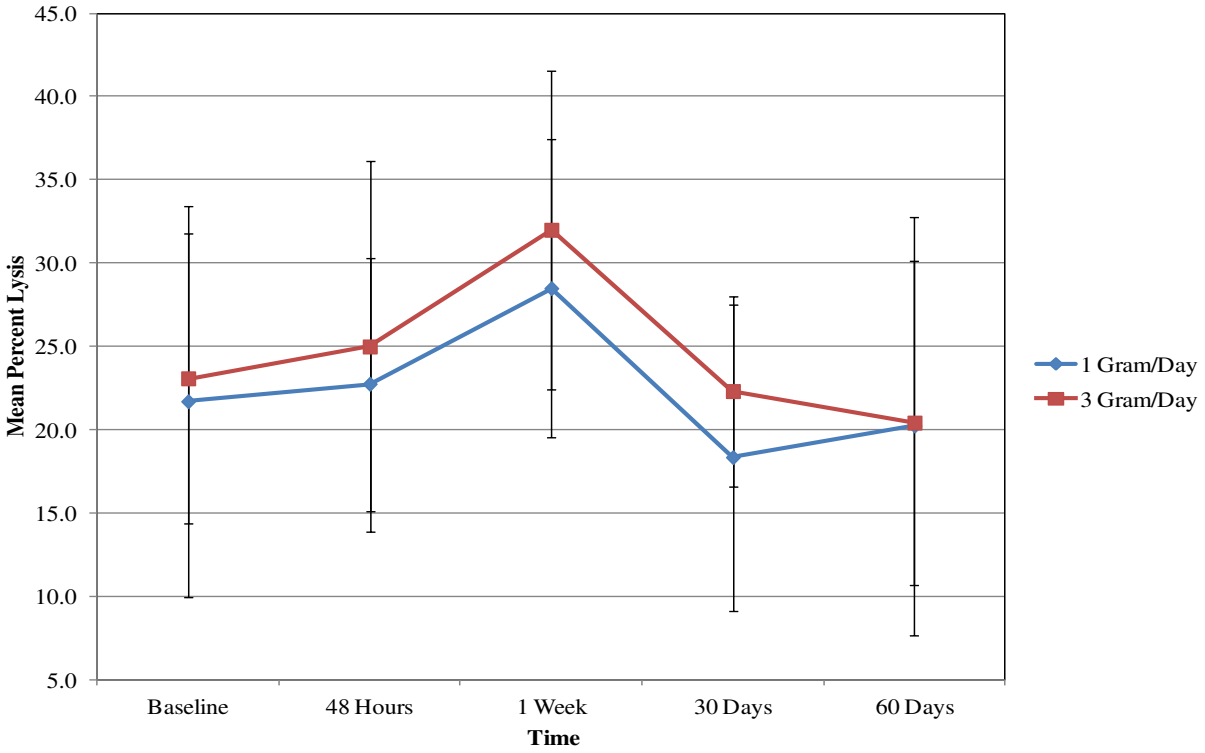

Figure 1. NK Cell Cytotoxicity from Baseline to 60 Days

NKCC (Percent Lysis) at Baseline, 48 Hours, 1 Week, 30 Days, and 60 Days was measured by flow cytometric assay. As shown and described in the text, NKCC peaked at 1 week and was significantly higher than all other values and even declined below the baseline value at the 30 and 60 day follow-up assessments.

Cytokines and Growth Factors. Table 2 shows the descriptive values for all 12 cytokines and growth factors at baseline, 1 week, 30 days, and 60 days for the entire sample and both groups. We found no significant effects for IL-4 and IL-8.

Table 2. Cytokines and Growth Factors at Baseline through 60 Days

Measure	Time	Total Sample (n=20)	1 Gram/Day (n=10)	3 Gram/Day (n=10)
IL-2 (pg/mL)	Baseline	3.94±4.04 (0.00, 17.85)	3.33±5.46 (0.00, 17.85)	4.56±1.96 (3.29, 9.96)
	1 Week	2.65±2.62 (0.00, 9.61)	3.47±2.97 (0.00, 9.61)	1.83±2.03 (0.00, 4.55)
	30 Days	3.31±2.42 (0.00, 7.07)	3.60±2.67 (0.00, 7.07)	3.02±2.26 (0.00, 6.64)
	60 Days	5.53±2.74 (0.00, 9.51)	5.51±3.17 (0.00, 9.51)	5.55±2.40 (0.00, 9.18)
IL-4 (pg/mL)	Baseline	1.50±1.68 (0.00, 6.84)	1.85±2.15 (0.00, 6.84)	1.16±1.02 (0.00, 2.54)
	1 Week	1.93±2.90 (0.00, 12.48)	2.65±3.74 (0.00, 12.48)	1.21±1.63 (0.00, 4.20)
	30 Days	1.49±1.23 (0.00, 3.65)	1.57±1.47 (0.00, 3.65)	1.41±1.02 (0.00, 2.78)

	60 Days	0.75±1.74 (0.00, 6.88)	1.49±2.28 (0.00, 6.88)	0.00±0.00 (0.00, 0.00)
IL-6 (pg/mL)	Baseline	0.90±1.10 (0.00, 5.21)	1.22±1.41 (0.58, 5.21)	0.57±0.76 (0.00, 1.41)
	1 Week	0.48±0.70 (0.00, 2.89)	0.71±0.90 (0.00, 2.89)	0.26±0.37 (0.00, 0.87)
	30 Days	0.82±0.48 (0.00, 1.48)	0.89±0.38 (0.00, 1.30)	0.75±0.57 (0.00, 1.48)
	60 Days	0.40±0.67 (0.00, 2.15)	0.53±0.75 (0.00, 2.15)	0.27±0.59 (0.00, 1.66)
IL-8 (pg/mL)	Baseline	6.50±7.74 (1.92, 35.60)	7.74±10.18 (2.30, 35.60)	5.26±4.43 (1.92, 16.90)
	1 Week	3.53±3.36 (0.00, 13.80)	3.74±4.14 (0.00, 13.80)	3.31±2.58 (0.00, 6.90)
	30 Days	6.81±6.93 (0.00, 25.10)	6.49±3.44 (0.00, 10.90)	7.12±9.45 (0.00, 25.10)
	60 Days	3.13±3.82 (0.00, 12.20)	2.67±3.82 (0.00, 12.20)	3.60±3.97 (0.00, 9.02)
IL-10 (pg/mL)	Baseline	0.67±1.32 (0.00, 5.76)	0.49±0.66 (0.00, 1.75)	0.85±1.78 (0.00, 5.76)
	1 Week	0.22±0.32 (0.00, 0.90)	0.21±0.35 (0.00, 0.90)	0.23±0.30 (0.00, 0.60)
	30 Days	0.83±0.55 (0.00, 2.12)	1.05±0.51 (0.00, 2.12)	0.60±0.52 (0.00, 1.19)
	60 Days	0.25±0.83 (0.00, 3.44)	0.15±0.49 (0.00, 1.54)	0.34±1.09 (0.00, 3.44)
IL-1α (pg/mL)	Baseline	0.56±0.51 (0.00, 1.92)	0.63±0.57 (0.00, 1.92)	0.48±0.46 (0.00, 1.13)
	1 Week	0.13±0.24 (0.00, 0.90)	0.16±0.30 (0.00, 0.90)	0.11±0.18 (0.00, 0.49)
	30 Days	0.64±0.56 (0.00, 2.28)	0.90±0.59 (0.00, 2.28)	0.38±0.42 (0.00, 1.00)
	60 Days	0.14±0.28 (0.00, 0.80)	0.14±0.29 (0.00, 0.80)	0.13±0.28 (0.00, 0.73)
IL-1β (pg/mL)	Baseline	3.41±2.24 (0.00, 8.84)	3.51±1.99 (0.00, 6.87)	3.32±2.57 (0.00, 8.84)
	1 Week	1.69±2.54 (0.00, 8.60)	1.91±2.56 (0.00, 8.15)	1.46±2.63 (0.00, 8.60)
	30 Days	3.64±2.65 (0.00, 8.55)	3.63±2.19 (0.00, 7.02)	3.64±3.16 (0.00, 8.55)
	60 Days	0.49±1.22 (0.00, 4.11)	0.41±1.30 (0.00, 4.11)	0.57±1.21 (0.00, 3.19)
IFN-γ (pg/mL)	Baseline	0.56±1.04 (0.00, 3.39)	0.72±1.22 (0.00, 3.39)	0.40±0.86 (0.00, 2.21)
	1 Week	0.50±1.02 (0.00, 3.67)	0.53±0.90 (0.00, 2.46)	0.48±1.17 (0.00, 3.67)
	30 Days	1.86±1.89 (0.00, 7.09)	1.38±1.23 (0.00, 3.09)	2.35±2.35 (0.00, 7.09)
	60 Days	0.33±1.07 (0.00, 4.29)	0.43±1.36 (0.00, 4.29)	0.23±0.74 (0.00, 2.34)
TNF-α (pg/mL)	Baseline	1.78±0.88 (0.00, 3.00)	1.73±0.86 (0.00, 2.90)	1.84±0.95 (0.00, 3.00)
	1 Week	1.41±1.19 (0.00, 3.80)	1.34±1.32 (0.00, 3.80)	1.49±1.11 (0.00, 3.60)
	30 Days	1.79±1.49 (0.00, 6.19)	2.07±1.14 (1.03, 4.93)	1.51±1.80 (0.00, 6.19)
	60 Days	0.86±1.58 (0.00, 4.65)	0.67±1.44 (0.00, 3.90)	1.05±1.76 (0.00, 4.65)
MCP-1 (pg/mL)	Baseline	60.72±21.88 (21.71, 103.70)	63.58±19.93 (37.28, 103.70)	57.86±24.41 (21.71, 92.66)
	1 Week	71.48±24.85 (22.01, 113.17)	73.17±26.12 (42.64, 113.17)	69.79±24.80 (22.01, 101.67)
	30 Days	81.86±41.75 (29.15, 183.97)	83.27±33.02 (37.72, 130.36)	80.46±50.85 (29.15, 183.97)
	60 Days	86.45±25.94 (38.93, 138.57)	84.14±14.38 (52.97, 102.34)	88.77±34.66 (38.93, 138.57)
VEGF (pg/mL)	Baseline	45.13±35.96 (12.47, 142.40)	41.19±30.29 (13.41, 97.91)	49.06±42.16 (12.47, 142.40)
	1 Week	29.27±32.00 (0.00, 126.02)	18.98±19.64 (0.00, 51.95)	39.56±39.23 (0.00, 126.02)
	30 Days	40.28±38.33 (0.00, 159.40)	42.42±33.27 (12.94, 120.20)	38.13±44.54 (0.00, 159.40)
	60 Days	24.06±23.66 (0.00, 82.56)	16.64±13.06 (0.00, 38.65)	31.47±29.82 (0.00, 82.56)
EGF (pg/mL)	Baseline	32.90±41.90 (4.33, 174.94)	27.14±27.62 (6.88, 93.65)	38.66±53.58 (4.33, 174.94)
	1 Week	11.62±14.35 (0.00, 60.31)	8.06±8.60 (1.55, 22.60)	15.18±18.23 (0.00, 60.31)
	30 Days	53.49±52.67 (2.18, 211.90)	58.86±41.19 (2.18, 127.13)	48.13±64.00 (3.67, 211.90)
	60 Days	14.70±12.29 (0.00, 48.73)	13.76±10.18 (2.80, 31.74)	15.64±14.61 (0.00, 48.73)

NOTE: Values are mean ± standard deviation (minimum, maximum).

For IL-2, a significant effect was found for time ($F[2.7,47.7] = 3.3$, $p = 0.037$), but the group by time interaction ($p = 0.51$) and the main effect of group ($p = 0.72$) were non-significant. The ώ degrees of freedom Huynh-Feldt correction factor for the within-subjects effects was 0.884. Pairwise comparisons revealed that the value at 60 days (M = 5.53; SE = 0.63, 95% CI: 4.21,

6.85) was significantly higher than the 1 week (M = 2.65; SE = 0.57, 95% CI: 1.45, 3.85; p = 0.001) and 30 day (M = 3.31; SE = 0.55, 95% CI: 2.15, 4.47; p = 0.016) values. No other values were significantly different.

For IL-6, a significant effect was found for time (F[2.6,47.5] = 3.5, p = 0.028), but the group by time interaction (p = 0.53) and main effect of group (p = 0.15) were non-significant. The ώ degrees of freedom Huynh-Feldt correction factor for the within-subjects effects was 0.880. For the total sample, IL-6 decreased at 1 week, increased at 30 days, and decreased again at 60 days, and further pairwise comparisons revealed that the value at baseline (M = 0.90; SE = 0.24, 95% CI: 0.39, 1.40) was significantly higher than the 1 week (M = 0.48; SE = 0.15, 95% CI: 0.16, 0.80; p = 0.027) and 60 day (M = 0.40; SE = 0.15, 95% CI: 0.08, 0.72; p = 0.014) values, and the 30 day value (M = 0.82; SE = 0.11, 95% CI: 0.60, 1.05) was significantly higher than the 60 day value (p = 0.013). No other values were significantly different.

For IL-10, a significant effect was found for time (F[1.5,27.4] = 3.9, p = 0.04), but the group by time interaction (p = 0.28) and main effect of group (p = 0.91) were non-significant. The ώ degrees of freedom Huynh-Feldt correction factor for the within-subjects effects was 0.507. Pairwise comparisons revealed that the value at 1 week (M = 0.22; SE = 0.73, 95% CI: 0.07, 0.38) was significantly lower than the 30 day value (M = 0.83; SE = 0.12, 95% CI: 0.58, 1.07), and the 60 day value (M = 0.25; SE = 0.19, 95% CI: -0.15, 0.65) was significantly lower than the baseline (M = 0.67; SE = 0.30, 95% CI: 0.04, 1.30; p = 0.009) and 30 day (p = 0.007) values. No other values were significantly different.

For IL-1α, a significant effect was found for time (F[2.2,40.4] = 11.7, p = 0.001), but the group by time interaction (p = 0.12) and main effect of group (p = 0.16) were non-significant. The ώ degrees of freedom Huynh-Feldt correction factor for the within-subjects effects was 0.748. Pairwise comparisons revealed that the value at baseline (M = 0.56; SE = 0.12, 95% CI: 0.31, 0.80) was significantly higher than at 1 week (M = 0.13; SE = 0.06, 95% CI: 0.02, 0.25; p < 0.001) and at 60 days (M = 0.14; SE = 0.06, 95% CI: 0.01, 0.27; p < 0.001), and the value at 30 days (M = 0.64; SE = 0.11, 95% CI: 0.40, 0.88) was significantly higher than at 1 week (p = 0.001) and at 60 days (p = 0.001). No other values were significantly different.

For IL-1β, a significant effect was found for time (F[2.7,48.2] = 11.8, p = 0.001), but the group by time interaction (p = 0.95) and main effect of group (p = 0.87) were non-significant. The ώ degrees of freedom Huynh-Feldt correction factor for the within-subjects effects was 0.893. Pairwise comparisons revealed that the value at 1 week (M = 1.69; SE = 0.58, 95% CI: 0.47, 2.91) was significantly lower than at baseline (M = 3.41; SE = 0.51, 95% CI: 2.33, 4.49; p = 0.009) and at 30 days (M = 3.64; SE = 0.61, 95% CI: 2.36, 4.91; p = 0.03) and significantly higher than at 60 days (M = 0.49; SE = 0.28, 95% CI: -0.10, 1.08; p < 0.04). The value at 60 days was significantly lower than at baseline (p < 0.001) and 30 days (p < 0.001). No other values were significantly different.

For IFN-γ, a significant effect was found for time (F[2.0,36.6] = 6.9, p = 0.003), but the group by time interaction (p = 0.32) and main effect of group (p = 0.78) were non-significant. The ώ degrees of freedom Huynh-Feldt correction factor for the within-subjects effects was 0.677. IFN-γ peaked at the 30-day assessment (M = 1.86; SE = 0.42, 95% CI: 0.98, 2.75), which was significantly higher than the baseline (M = 0.56; SE = 0.24, 95% CI: 0.06, 1.06; p = 0.017),

1 week (M = 0.50; SE = 0.23, 95% CI: 0.01, 1.00; p = 0.012), and 60 day (M = 0.33; SE = 0.24, 95% CI: -0.18, 0.85; p = 0.003) values. No other values were significantly different.

For TNF-α, a significant effect was found for time (F[2.9,51.6] = 3.8, p = 0.017), but the group by time interaction (p = 0.48) and main effect of group (p = 0.97) were non-significant. The ώ degrees of freedom Huynh-Feldt correction factor for the within-subjects effects was 0.956. Pairwise comparisons revealed that the value at 60 days (M = 0.86; SE = 0.36, 95% CI: 0.11, 1.62) was significantly lower than the baseline (M = 1.78; SE = 0.20, 95% CI: 1.36, 2.21; p = 0.012) and 30 day (M = 1.79; SE = 0.34, 95% CI: 1.08, 2.50; p = 0.037) values. No other values were significantly different.

For MCP-1, a significant effect was found for time (F[2.1,37.8] = 3.9, p = 0.026), but the group by time interaction (p = 0.87) and main effect of group (p = 0.85) were non-significant. The ώ degrees of freedom Huynh-Feldt correction factor for the within-subjects effects was 0.700. MCP-1 peaked at 60 days (M = 86.45; SE = 5.93, 95% CI: 73.98, 98.92) and was significantly higher than the baseline (M = 60.72; SE = 4.98, 95% CI: 50.25, 71.18; p = 0.001) and 1 week (M = 71.48; SE = 5.70, 95% CI: 59.51, 83.44; p = 0.05) values. No other values were significantly different.

For VEGF, a significant effect was found for time (F[2.6,47.3] = 4.8, p = 0.007), but the group by time interaction (p = 0.38) and main effect of group (p = 0.45) were non-significant. The ώ degrees of freedom Huynh-Feldt correction factor for the within-subjects effects was 0.877. Pairwise comparisons revealed that the value at baseline (M = 45.13; SE = 8.21, 95% CI: 27.88, 62.37) was significantly higher than the 1 week (M = 29.27; SE = 6.94, 95% CI: 14.70, 43.85; p = 0.02) and 60 day (M = 24.06; SE = 5.15, 95% CI: 13.24, 34.87; p = 0.007) values. No other values were significantly different.

For EGF, a significant effect was found for time (F[2.1,38.0] = 7.8, p = 0.001), but the group by time interaction (p = 0.63) and main effect of group (p = 0.82) were non-significant. The ώ degrees of freedom Huynh-Feldt correction factor for the within-subjects effects was 0.703. Pairwise comparisons revealed that the value at baseline (M = 32.90; SE = 9.53, 95% CI: 12.88, 52.92; p = 0.034) was significantly higher than 1 week (M = 11.62; SE = 3.19, 95% CI: 4.92, 18.32) and 60 days (M = 14.70; SE = 2.82, 95% CI: 8.79, 20.61). The value at 30 days (M = 53.49; SE = 12.03, 95% CI: 28.21, 78.78) was significantly higher than at 1 week (p = 0.001) and 60 days (p = 0.004). No other values were significantly different.

DISCUSSION

The ability of individuals to defend against infectious diseases and restrict the progression of carcinogenesis depends upon both immunity and nutritional factors. Immune surveillance is an important host protection function, specifically as it serves to inhibit the progression of carcinogenesis and viral infection. Several immune effector cells and secreted cytokines play critical roles in the overall process. The enhancement of NKCC by RBAC is expected to translate into enhanced immune surveillance.

In the current study, RBAC demonstrated significant immunomodulatory activity based upon the observed changes in NKCC and the cytokines and growth factors. Our results are consistent with prior work by other investigators on RBAC showing this product's ability to

positively impact immune system functioning [6-8]. RBAC action was rapid enough to significantly enhance NKCC within 1 week of administration in RBAC-naïve adult subjects. Following its peak at 1 week, NKCC declined to baseline levels by 30 days. The increase in NKCC appears to be due to an increase in NK activity on a per-cell basis, rather than due to an increase in the actual NK cell number. This is supported by our finding that lower E:T ratios of 12.5:1 and 25:1 were highly effective against K562 cell lysis.

Nine out of 12 cytokines and growth factors showed changes over time for the total sample (no difference between groups), specifically between baseline, 1 week, and 30 and 60 days. The Th1 cytokine profile showed that IL-2, IFN-γ, and TNF-α decreased at 1 week, but significantly increased at 30 days for IFN-γ and TNF-α and at 60 days for IL-2. Most of the Th2 cytokines, including IL-10, showed an initial decline at 1 week and then returned to baseline levels by 30 days similar to the trend observed with Th1 cytokines.

IL-4 was the only cytokine that increased in the first week of RBAC administration. This period corresponds directly with the time course of the NKCC spike. As IL-4 is a potent regulator of NKCC, the potential exists that the NKCC enhancing effect of RBAC is IL-4-mediated. MCP-1 was the only cytokine that continuously increased during the intervention. MCP-1 is a pro-inflammatory chemokine, functioning as a chemoattractant and NK cell activator [14;15]. MCP-1 has been shown to induce migration of activated NK cells *in vitro* [16]. RBAC promotion of MCP-1 release may act to enhance NK cell access to sites of infection and/or malignant proliferation. Additional studies are needed to assess the potential of selective upregulation of effector cell chemokine receptors following RBAC intake.

It is important to note that in our study of RBAC in healthy adults we detected responses that were not solely immunostimulatory or immunosuppressive. Rather, our trial showed transient, bi-directional immune marker effects consistent with true multifactorial immunomodulation. When given an immunomodulator, it may be postulated that the immune system becomes alert to the existing infections and disorders within the body. In a healthy normal adult, the infectious and toxic presence within the body may be small enough that an optimally functioning immune system can eliminate it relatively quickly. Therefore, the NKCC and other immune activation parameters would show only a transient enhancement. In patients with functionally-compromised immunity (e.g., due to environmental factors or lifestyle habits) or serious illnesses (e.g., cancer, HIV/AIDS, or other life-threatening infections), the immune system response to an immunomodulator would need to be prolonged due to the greater malignant, infectious, and toxic burdens present [17].

The importance of NKCC in controlling the progression of carcinogenesis is exemplified by the finding that some tumors employ mechanisms to confound NK immunosurveillance, e.g., production of decoy NKG2D receptor ligands [4]. Additionally, it has been established that depressed NKCC is associated with increased cancer risk; however the specific mechanisms of immunomodulation remain uncertain [18]. Development of effective therapeutic strategies will require great work in the clarification of NKCC *in vitro* and *in vivo*; especially in the identification of specific NK receptor-activating ligands.

Investigators focused on bioactive natural products have identified a variety of polysaccharides that possess immunomodulator activity [19]. Clinical and laboratory studies

have shown both innate and adaptive immune system effects, and polysaccharide receptors have been found on NK cells, T- and B-lymphocytes, macrophages, monocytes, and other effector cells [20]. Thus, RBAC fractions or isolates may potentially contain NK receptor ligands or analogous molecular species that could be used as experimental probes. Of note, the oligosaccharides present in the RBAC complex bear structural homology with sialyl Lewis x residues, which are known NK cell lectin-like receptor ligands and activators of NKCC [21;22]. Additionally, oligosaccharide moieties may function as ligands for natural cytotoxicity receptors [23].

Research efforts are underway in several centers to exploit NK cell functions to improve the management of both cancer patients and patients at high-risk for the development of malignancies [24]. Therapeutic strategies combining agents that activate anti-tumor immune responses with cytotoxic chemotherapeutic agents may enhance cancer treatment outcomes. Furthermore, IL-2 produces an augmentation in NKCC in a manner similar to RBAC, and combining the two agents may result in an additive or synergistic effect useful for the treatment of malignant melanoma and renal cell carcinoma. This is of particular interest, as it may allow for a decrease in the dosage of IL-2, thereby reducing toxicity without compromising therapeutic efficacy. Further elucidation of the cellular and molecular mechanisms involved in anti-tumor immune responses will be needed.

Given that metastasis is the cause of 90% of cancer deaths in humans [25], RBAC or similar immunomodulators may allow for the development of truly preventive anti-cancer strategies by optimizing NKCC. This could potentially enable transformed cells at clinically-undetectable stages of carcinogenesis and circulating tumor cells (CTCs) to be eliminated prior to their development into primary or metastatic tumors. This carries significant potential to improve outcomes in clinical and surgical oncology, where patients have existent CTCs and/or develop CTCs secondary to mechanical disturbance of malignant tissues [26;27].

Thus, the results of our study suggest that RBAC immunomodulation merits further investigation in humans; specifically in the presence of conditions where the immune system is challenged and/or functionally-compromised, e.g., tobacco smoking, influenza, HIV/AIDS, and cancer. We anticipate that RBAC may: (a) facilitate the destruction of malignant and virus-infected cells, (b) enhance immune effector cell activity, (c) optimize immune system function, and (d) improve host recovery, long-term health maintenance, and overall quality of life.

Limitations. The findings of this study are limited by a small sample size. A larger sample size could result in even more significant findings for NKCC, cytokines, and growth factors in addition to further strengthening the distinction in the results between the two dosage groups. It was not possible to objectively determine compliance with the protocol, as the composition of the RBAC complex does not readily lend itself to metabolite assessment. Although we found the highest peak in NKCC at 1 week and in most of the cytokines and growth factors at 30 days, a follow-up period of 6 to 12 months with continued RBAC administration could elucidate the ability of RBAC to modulate immune system function over an extended period of time.

CONCLUSIONS

In summary, RBAC was well-tolerated among all subjects. All subjects completed every

assessment without reporting any adverse event, and the assessments were completed in a timely fashion. The supplement was typically safe among subjects in both dosage groups according to measures of creatinine, total protein, total bilirubin, ALT, AST, and ALP. RBAC demonstrated sound immunomodulatory activity with significant responses in NKCC and nine out of twelve cytokines and growth factors. No statistical differences were found between the two dosage groups in the measured parameters. Therefore, the two dosages recommended by the manufacturer of RBAC (i.e., 1 gram/day and 3 gram/day) would appear equivalent with respect to the observed effects on NKCC, cytokines, and growth factors in healthy adults.

Our study showed transient, bidirectional, immune marker effects consistent with true multifactorial immunomodulation. An important tendency noted in the activity profiles of several natural product immunomodulators is that of "optimization" or true immunomodulation, rather than simple upregulation or downregulation of the immune response. Thus, a healthy individual's response to an immunomodulator is expected to be distinctly different from the response during an illness like influenza, HIV/AIDS, or cancer.

More studies are required to compare the role of NKCC and the cytokine and growth factor profile following RBAC intake to further delineate the selective and sustained NK activation property of RBAC. Finally, the results of this study in healthy subjects suggest that the immunomodulatory activity of RBAC should be studied in conditions where the human immune system has been functionally-compromised: e.g., tobacco smoking, cancer, HIV/AIDS, etc. RBAC may not only facilitate destruction of tumor and virus-infected cells, but may also increase immune effector cell activity, and it may enhance host recovery, long-term health maintenance, and overall quality of life by optimizing immune system function.

List of Abbreviations

natural killer (NK), NK cell cytotoxicity (NKCC), interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), monocyte chemotactic protein-1 (MCP-1), interleukins (IL) 2-10, rice bran arabinoxylan compound (RBAC), alanine transaminase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), dioctadecyloxacarbocyanine perchlorate (DiO), complete medium (CM), propidium iodide (PI), effector-to-target (E:T), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), analysis of variance (ANOVA), standard deviation (SD), mean (M), standard error (SE), confidence interval (CI), and circulating tumor cells (CTCs)

Competing Interests: Karriem H. Ali has received income from Daiwa Pharmaceutical Inc., as a member of its scientific advisory board. No other authors have any competing interests to report.

Authors' Contributions: Drs. Ali, Asthana, Woolger, Wolfson, McDaniel, and Lewis, and Mses. Melillo and Leonard contributed to the design of the study. Drs. Ali, Asthana, Woolger, Wolfson, McDaniel, and Lewis contributed to the writing of the article. Drs. Ali, Asthana, Wolfson, and Lewis, and Mses. Melillo and Leonard contributed to the analysis of the data.

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