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# Comparison of immunological properties of various bioactive combinations - Part II

## Abstract

**Aim:** More and more various mixtures of potentially bioactive molecules reach the market, however the serious testing is usually lacking.

**Methods:** In this study we directly compared the effects of eleven different combinations on the immune reactions.

**Results:** We evaluated phagocytic activity, IL-2 and superoxide anion formation, NK cell activity, antibody response and breast cancer inhibition. Our results demonstrated strong differences among individual combinations.

**Conclusion:** In most cases, these combinations have no effects at all. The only consistently active mixture was RVB 300.

**Keywords:** phagocytosis, IL-2, NK cells, antibodies, cancer, immunity, β-glucan, vitamin C, immunomodulators

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## Introduction

Biologically active, safe and most of all natural stimulators of the immune system have been sought throughout history. Some of those, like β-glucan, are intensively studied and now, over 15,000 publications later, are being used in numerous clinical trials[1] and in some countries already represent an established drug.[2] However, the search for even better effects continues. Along with the testing of new natural molecules, more and more manufacturers are offering various cocktails or mixtures with potential immunostimulating properties. The main problem with these combinations is the limited research that would support the healthy claims. Individual components are often biologically active or at least have established effects on human health (in the case of vitamins and minerals), but the effects of these combinations are rarely tested. In these cases, there can be dozens of different ingredients and we have absolutely no clue if they have any synergistic activities.

The idea of these combinations is often based on old folks remedies, with limited if any scientific background. Some substances might have no activity, some might stimulate, and some might even inhibit the immune system. Another problem is based on fact that the description of these mixtures uses vague terms such as extract or proprietary blend, which offer no information at all. Despite the fact that these combinations might have questionable qualities, it is important to note that, studies showing that some bioactive molecules have synergistic effects with others exist. In the case of glucan, numerous scientific studies have shown beneficial effects when glucan was given in combination with vitamin C,[3] humic acid[4] and Resveratrol.[5] Natural immunomodulators are slowly becoming a mainstream supplement and, with dozens of clinical trials of some of these molecules under way, their use in regular clinical practice might only be a question of time. There is extremely limited number of scientific studies of individual combinations with supposedly immunostimulating properties. One study directly describing immunological effects of six such commercially popular combinations found that only one of them had any significant effects.[6] In this follow up study, we decided to use 10 additional combinations and compare them against RVB 300, which was the most active combination in the original study.

## Material and methods

### Animals

Female, 8 week old BALB/c mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All animal work was done according to the University of Louisville IACUC protocol. Animals were sacrificed by $CO_2$ asphyxiation.

### Material

Individual samples were purchased from the manufacturers or distributors as shown in Table 1. RPMI 1640 medium, sodium citrate, Wright stain, Concanavalin A, HEPES, PMA, Cytochrome C, penicillin and streptomycin were obtained from Sigma Chemical Co. (St. Louis, MO). Fetal calf serum (FCS) was from Hyclone Laboratories (Logan, UT).

### Cell lines

Human neutrophil cell line HL-60 and YAC cells were obtained from the ATCC (Manassas, VA). The BALB/c mouse-derived mammary tumor cell line Ptac64 was generously provided by Dr. Wei-Zen Wei of the Michigan Cancer Foundation, Wayne State University, and Detroit, MI. The cell lines were maintained in RPMI 1640 medium containing HEPES buffer supplemented with 10% heat-inactivated FCS, 100U/ml penicillin and 100μg/ml streptomycin, in plastic disposable tissue culture flasks at 37°C in a 5% $CO_2$/95% air incubator.

### Phagocytosis

The technique employing Phagocytosis of synthetic polymeric microspheres was described in Vetvicka et al.[7,8]

**Briefly:** peripheral blood cells were incubated *in vitro* with 0.05ml of 2-hydroxyethyl methacrylate particles (HEMA; $5x10^8$/ml). The test

 

tubes were incubated at 37°C for 60min., with intermittent shaking. Smears were stained with Wright stain. The cells with three or more HEMA particles were considered positive. Mice were injected ip with individual samples or PBS (control). All experiments were performed in triplicate. At least 200 cells in 60 high power fields were examined in each experiment.

### IL-2 production

Purified spleen cells ($2x10^6$/ml in RPMI 1640 medium with 5% FCS) from mice injected with tested samples were added into wells of a 24-well tissue culture plate. After addition of 1mg of Concanavalin A (positive control), cells were incubated for 48hrs in a humidified incubator (37°C, 5% $CO_2$). At the endpoint of incubation, supernatants were collected, filtered through 0.45µm filters and tested for the presence of IL-2 using a Quantikine mouse IL-2 kit (R&D Systems, Minneapolis, MN).

### Antibody formation

Formation of antibodies was evaluated using ovalbumin as an antigen. Mice were injected twice (two weeks apart) with 0.1mg of ovalbumin and the serum was collected 7days after last injection. Experimental groups were getting daily ip. injections of tested material. The level of specific antibodies against ovalbumin was detected by ELISA. As a positive control, a combination of ovalbumin and Freund's adjuvant was used.

### Superoxide and nitrite production

Cells were incubated in a final volume of 200µl of medium containing 0.1% gelatin and 100µM Cytochrome C. Mice were challenged with 100µg of individual glucans 24hrs earlier. Cell lines were incubated with 1µg/ml of glucans for 24hrs. For the superoxide production, the reaction was initialized by the addition of 5ng/ml PMA. After gentle mixing, the absorbance was measured 30minutes after incubation at 37°C using multi well spectrophotometer at 550nm. Results are expressed as nanomoles of cytochrome C reduced/$2.5x10^5$cells/30 minutes, after subtraction of the SOD and spontaneous release controls.[9]

### In vitro cytotoxicity assay

Spleen cells were isolated from spleen of mice by standard methods. Cell suspension was generated by pressing minced spleen against the bottom of a Petri dish containing PBS. After elimination of erythrocytes by 10-second incubation in distilled water, and five washes in cold PBS, the cells were resuspended in PBS and counted. The viability was determined by trypan blue exclusion. Only cells with viability better than 95% were used in subsequent experiments. Splenocytes ($10^6$/ml; 0.1ml/well) in V-shaped 96-well microplates were incubated with individual samples (2µg/ml) for 30min at 37°C and then washed three times with RPMI 1640 medium. After washing, 50µl of target cell line YAC-1 (two different concentrations of target cells were used so the final effector-target ratio was 32:1 and 64:1). After spinning the plates at 250xg for 5min, the plates were incubated for 4hrs at 37°C. The cytotoxic activity of cells was determined by the use of CytoTox96 Non-Radioactive Cytotoxicity Assay from Promega (Promega, Madison, WI, USA) according to the manufacturer's instructions. Briefly, 10µl of lysis solution was added into the appropriate control wells 45min before the end of incubation. The next step was to spin the plates at 250xg for 5min, followed by transferring 50µl of supernatant into flat-bottomed, 96-well microplates. After 50µl of reconstituted substrate was added into each well, plates were covered and incubated for 30min at room temperature at dark. The optical density was determined by using a STL ELISA reader (Tecan U.S., Research Triangle Park, NC) at 492nm. Specific cell-mediated cytotoxicity was calculated using the formula:

Percent-specific killing (% cytotoxicity)=100x[($OD_{492}$ experimental-$OD_{492}$ spontaneous) divided ($OD_{492}$ maximum-$OD_{492}$ spontaneous)] as described in manufacturer's instructions, where spontaneous release was target cells incubated with medium alone and maximum release was that obtained from target cells lysed with the solution provided in the kit.

### Breast cancer model

Mice were injected directly into the mammary fat pads with $1x10^6$/mouse of Ptas64 cells in PBS. The experimental treatment was begun after palpable tumors were found (usually 14 days after injection of cells) and after mice were assigned to experimental groups. Experimental treatment was achieved by intraperitoneal injections of tested samples diluted in PBS (once/day for 14days). After treatment, the mice were sacrificed, tumors removed and weighed.[10]

## Results

The number of various combinations of natural immunomodulators is steadily increasing. The rationale for their formulation is usually a combination of marketing tricks with honest efforts to prepare the immunomodulator with optimal biological effects. For our study, we picked eleven easily available, commercially successful combinations, claiming significant immunostimulating effects. Information about individual formulations and manufacturers is given in Table 1. Phagocytosis is one of the most important reactions regulating the primary defense against invading microorganisms. We used a well-established technique employing synthetic microspheres. Our results summarized in Table 2 showed that most samples showed little (if any) activity. Only two samples showed significant improvements - Killer Biotic only at the highest dose, 7M from a dose of 100µg and RVB 300 throughout the whole spectrum of doses.

In addition to Phagocytosis, oxidative burst represents another important step in innate immunity. Table 3 shows the effect of tested samples on production of superoxide anion by human neutrophil cell line HL-60. As the production of superoxide anion after PBS application is very low (0.13 nanomoles/$2.5x10^5$cells), we found more positive samples. However, even four positive samples (Biotic Killler, 7M, MGB 3 and Avemar) combined did not reach the effects of RVB 300.

Next we focused on the effects of our samples on production of IL-2 by cells isolated from spleen. The levels of IL-2 were measured after 48hrs of *in vitro* incubation of splenocytes. PBS stimulated only negligible levels (1.3pg/ml) of IL-2, Concanavalin A as positive control levels were in 2,400 to 2,600pg/ml ranges). With such low control levels, it is not surprising that all samples showed statistically significant effects. However, only RVB 300(1,001pg/ml) and 7M (223.7pg/ml) effects can be considered strong (Table 4). NK cells are a type of lymphocytes and play a major role in the host-rejection of both tumors and virally infected cells. Our comparisons of the effects of various samples on killing of YAC-1 cells by NK cells showed that only RVB 300, Avemar and 7M had significant activity (Table 5).

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Next, we tested the possible role of our materials in influencing the antibody response. Using an experimental model of mice immunized with ovalbumin (and Freund's adjuvant as a positive control), we found three samples with positive effects - 7M, Avemar and RVB 300 (Table 6). The last part of our study was devoted to testing the effects of various components on cancer growth. The samples were used daily for 14days after the injected cancer cells showed visible growth (10-14days after application). Table 7 summarizes the results of two weeks of treatment on weight of tumors. Killer Biotic caused 22% inhibition of cancer growth, 7M caused 39% inhibition, MGN 334% inhibition and Avemar caused 29% inhibition. Compared to these data, inhibition caused by RVB 300(53 percent) was much higher.

**Table 1** Type of combination used in this study

| Sample | Composition | Source |
|---|---|---|
| Carnivora | Carnivora | Carnivora Research Int., Weston, CT, USA |
| Killer Biotic FX | Colostrum<br>Beta Sitosterol<br>Olive Leaf extract<br>Propolis concentrate Cayanne fruit concentrate<br>Garlic concentrate<br>Ginger root extract Cordyceps sinensis<br>Coriolus versicolor<br>Maitake mushroom extract<br>Reishi mushroom extract<br>Shitake mushroom extract | Youngevity, Chula Vista |
| Beta Immune Booster | Dried Yeast fermentate<br>Vitamins (A,C,E)<br>Mg, Se, Zn, Mn, Na, K | Reckit Benckiser Parsippany, NJ, USA |
| 7M Complete Immune Booster | Brown seaweed extract<br>Humic acid<br>Fulvic acid<br>Proprietary blend of mushrooms | Epigenetic Labs Centennial, CO, USA |
| Emergen-C | P, K, Zn, Mn, Cr, Na, K<br>Vitamin C, B1, B3, B5, B6, B9,B12 | Alacer Corp., Foothill Ranch, CA, USA |
| Immortalium | Vitamin A, C, $D_3$, E, K, $B_6$, Zn, Cu MSM Alpha<br>Lipoic acid Telomere support blend<br>Trans resveratrol<br>Whole Food Blend Fuciodan-rich algae blend<br>Regeneryll blend<br>Skin-rejuvenating blend<br>Skin-defense carotenoid blend | Youngevity, Chula Vista, CA, USA |
| Immu 911 | Vitamin C<br>Zn Proprietary Botanical blend | Youngevity, Chula Vista, CA, USA |
| MGN 3 | Arabinoxylan compound | Lane Labs, Allendale, NJ, USA |
| Avemar Bio-Immune | Avemar pulvis Ashwagandha<br>Boerhavia Indian Tinospora Extract Holy Basil<br>Gotu Kola<br>Vidanga<br>Neem<br>Dwarf Morning Glory<br>Indian Tinospora<br>Ginger<br>Long Pepper<br>Mica<br>Coral<br>Mesua | Avemar, Budapest, Hungary, VPK, Fairfield, IA, USA |
| RVB 300 | Glucan #300<br>Resveratrol<br>Vitamin C | Youngevity, Chula Vista, CA, USA |

**Table 2** Effects of various samples on phagocytosis

| Dose (mg/ml | 25 | 50 | 100 | 200 | 400 | 800 |
|---|---|---|---|---|---|---|
| Carnivora | 30.1±1.9 | 32.2±2.2 | 30.4±1.7 | 32.8±3.8 | 31.5±3.5 | 32.7±2.8 |
| Killer Biotic | 30.8±2.1 | 33.3±3.4 | 34.1±2.9 | 35.1±2.1 | 33.3±2.8 | 36.2±2.7* |
| Beta Immune Booster | 28.9±3.1 | 30.5±2.2 | 33.1±4.2 | 32.1±1.8 | 30.9±2.5 | 32.7±3.0 |
| 7M | 31.8±2.6 | 33.9±1.8 | 38.3±1.7* | 41.4±2.4* | 43.3±2.8* | 43.0±1.9* |
| Emergen-C | 28.9±1.9 | 30.0±2.5 | 29.5±1.8 | 32.1±2.7 | 31.8±2.6 | 33.3±3.7 |
| Immortalium | 30.6±2.5 | 31.7±2.8 | 32.2±1.9 | 31.7±2.8 | 32.9±1.1 | 33.1±2.9 |
| Immu 911 | 28.6±3.6 | 30.8±2.1 | 31.7±2.2 | 31.6±2.7 | 32.0±3.3 | 32.2±2.7 |
| MGN 3 | 30.5±1.9 | 31.8±2.2 | 32.7±1.8 | 33.5±2.1 | 32.8±3.8 | 33.5±2.9 |
| Avemar | 31.2±1.9 | 32.8±2.6 | 31.7±3.0 | 33.1±2.9 | 32.0±1.9 | 33.7±3.1 |
| Bio-Immune | 30.7±3.1 | 31.8±2.2 | 33.2 ±2.9 | 32.5±2.2 | 33.1±3.7 | 33.8±2.9 |
| RVB 300 | 39.8±2.2* | 45.3±4.4* | 65.5±2.8* | 72.2±3.6* | 75.1±3.8* | 72.5±4.0* |

Control values (PBS) were 30.3±2.7. The dose represents a single ip. Injection/mouse.

*Significant differences between sample and PBS at <0.05 level.

**Table 3** Effects of various samples on superoxide anion production

| Sample | Superoxide anion (nanomoles/2.5x105cells) |
|---|---|
| Carnivora | 0.21±0.03 |
| Killer Biotic | 0.58±0.21* |
| Beta Immune Booster | 0.22±0.06 |
| 7M | 0.55±0.11* |
| Emergen-C | 0.14±0.02 |
| Immortalium | 0.17±0.08 |
| Immu 911 | 0.15±0.02 |
| MGN 3 | 0.33±0.05* |
| Avemar | 0.25±0.05* |
| Bio-Immune | 0.20±0.07 |
| RVB 300 | 1.77±0.15* |
| PBS | 0.13±0.02 |

*Significant differences between sample and PBS at <0.05 level.

**Table 4** Effects of various samples on production of IL-2

| Sample | % Cytotoxicity |
|---|---|
| Carnivora | 18.2±1.8 |
| Killer Biotic | 68.6±5.5 |
| Beta Immune Booster | 36.3±2.5 |
| 7M | 223.7±11.2 |
| Emergen-C | 32.6±3.4 |
| Immortalium | 18.5±6.6 |
| Immu 911 | 32.0±2.2 |
| MGN 3 | 223.7±11.2 |
| Avemar | 32.6±3.4 |
| Bio-Immune | 18.5±6.6 |
| RVB 300 | 32.0±2.2 |

Control values (PBS) were 1.30.1.

*Significant differences between sample and PBS at <0.05 level.

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**Table 5** Effects of various samples on NK cell activity

| Sample | % Cytotoxicity |
|---|---|
| Carnivora | 11.1± 3.7 |
| Killer Biotic | 7.8±2.3 |
| Beta Immune Booster | 7.1±2.5 |
| 7M | 32.3±2.2* |
| Emergen-C | 11.1±3.7 |
| Immortalium | 7.8±2.3 |
| Immu 911 | 7.1±2.5 |
| MGN 3 | 7.4±1.1 |
| Avemar | 37.3±2.8* |
| Bio-Immune | 8.6±2.1 |
| RVB 300 | 45.7±3.3* |
| OVA | 6.6±0.9 |

*Significant differences between sample and PBS at <0.05 level.

**Table 6** Effects of various samples on formation of antibodies

| Sample | OD |
|---|---|
| Carnivora | 120.6±11.2 |
| Killer Biotic | 150.4±26.2 |
| Beta Immune Booster | 113.0±9.9 |
| 7M | 301.3±27.8* |
| Emergen-C | 109.6±6.6 |
| Immortalium | 133.6±21.5 |
| Immu 911 | 111.8±10.3 |
| MGN 3 | 92.9±9.7 |
| Avemar | 177.8±20.1* |
| Bio-Immune | 123.6±17.5 |
| RVB 300 | 483.2±41.3* |
| OVA | 101.1±18.2 |
| OVA + FA | 512.3±32.3* |

*Significant differences between sample and antigen alone (OVA) at P<0.05 level. Antigen and Freund's adjuvant (OVA + FA) served as a positive control.

Data are presented as mean ± SD.

**Table 7** Effects of various samples on cancer growth

| Sample | Weight of cancer (mg) |
|---|---|
| Carnivora | 612.7±52.1 |
| Killer Biotic | 513.6±41.1* |
| Beta Immune Booster | 660.1±43.3 |
| 7M | 398.9±34.1* |
| Emergen-C | 632.7±46.8 |
| Immortalium | 611.8±40.5 |
| Immu 911 | 622.7±21.8 |
| MGN 3 | 432.5±17.4* |
| Avemar | 468.3±38.4* |
| Bio-Immune | 599.9±38.5 |
| RVB 300 | 312.1±11.9* |
| PBS | 654.3 ± 47.7 |

*Significant differences between sample and PBS at <0.05 level.

## Discussion

Lately, the general disappointment with Big Pharma results in steadily increasing interests on natural molecules. Regardless of the fact that this trend is partly funded by a fear for chemicals in our life, by an unjustified push of various naturopaths or just by clever marketing, this trend is here to stay. Immunomodulator is the substance capable of interacting with the immune system resulting in up- or down-regulating specific parts of the immune response.[11] It is true that some natural immunomodulators, glucan in particular, have significant biological and physiological effects. It is understandable that a search for an even better molecule exists. However, the recent inclination to offer a better product often leads to questionable combinations, where manufacturers combine numerous biological extracts without any scientific support. In this study we decided to evaluate the question if more is really better. As numerous remedies have claims to improve our health via stimulation of the immune system, detailed studies confirming these possibilities are necessary. With the advanced understanding of immunology and ethnopharmacology, studies on the interaction of these products with individual segments of the immune system are critical to understand the potential efficacy. Careful selection of the correct targets for immunostimulation studies is imperative.[12]

The original study showed clear supremacy of RVB 300 (a combination of glucan, resveratrol and vitamin C) over five other combinations.[6] Samples tested in this study include arabinoxylan MGN 3 (known also under the name of Biobran), which is a cell modulator[13] with some synergy with curcumin.[14] Some studies even showed effects on cancer cells resulting in sensitization to paclitaxel effects.[15] Another study showed enhancement of NK cell activity towards neuroblastoma cells.[16] The seriousness of these findings is, however, somehow lowered by the fact that they almost entirely originate in the laboratory of the founder of MGN 3. Independent studies usually employed their own extract.[17] Avemar is fermented wheat germ extract developed by Mate Hidvegi with documented anti-carcinogenic properties.[18,19] Some of the effects are mediated via inhibition of the glycolysis enzymes and apoptosis stimulation.[20] In addition, an improvement in activity by the addition of vitamin C has been reported.[21] The rest of samples offered no scientific study with some exception of 7M Complete Immune Booster. There are studies showing strong synergistic effects of glucan and humic acid combination,[4,22] however these effects might depend on the quality and type of both glucan and/or humic acid, which clearly will be different in the present sample.

Our results clearly demonstrated strong differences among individual combinations. In most cases, these combinations have no effects at all. In the case of Phagocytosis, only three samples showed some activity, and these results were repeated in additional tests. Mostly, the combinations with some observed activities contained glucan, thus strongly suggesting that glucan is the component responsible for these actions. However, the activities were not too strong despite previously documented effects of these glucans, which leads to two possibilities - either the doses were not high enough or some parts of the combination actually suppressed the immune response. This is the main problem with these combinations-with no research on individual components, not to mention complete lack of research on their combination, we cannot even guess about possible interactions. In addition, so far there is no proof that combination of more than one glucan actually improves the effects.

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In all tests, the highest biological effects were found in case of RVB 300, which is a combination of glucan, resveratrol and vitamin C. Whereas in Phagocytosis and IL-2 productions, the effects are clearly caused by glucan, in oxidative burst they are probably caused by combination of glucan and vitamin C, as both components were described to stimulate oxidative burst.[23,24] Current trend to pile substances with potential or expected bioactive components is surely not optimal and can be even contra productive. Not only do we not know the final effects, but with various combinations showing no effects at all, it might have negative impact on the public and the whole industry, desperate to gain legitimacy and hoping to push through some of the supplements as official drug.

## Conclusions

It is important to note that it is imperative to carefully pick the right combination, as most of the tested combinations have only limited, if any, simulative effects on the immune system. The case of Youngevity, which on one hand offers products with superior effects (RVB 300) and on other hand offers products with no activity at all (Immortalium and Immune 911), suggests that we cannot pick the supplements based only on the manufacturer and his reputation.

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## Conflict of interest

The author declares no conflivt of interest.

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