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Full Length Research Paper

Synergistic effects of glucan and resveratrol

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Recent data showing that glucan stimulates defense reactions in plants through synthesis of resveratrol, led us to study the possible synergetic effects of a glucan-resveratrol complex on immune reactions in mice. We measured phagocytic activity, expression of CD4 marker on spleen cells, IL-2 secretion and antibody response. In all cases we confirmed the stimulatory effects of glucan. Resveratrol alone had either limit or has no effect. However, a combined preparation showed very strong synergetic effects. Our data support further studies of these two natural immunomodulators.

Key words: Glucan, resveratrol, phagocytosis, IL-2, immune reactions, macrophage, antibody.

INTRODUCTION

Glucans belong to a group of physiologically active compounds called biological response modifiers and represent highly conserved structural components of cell walls in yeast, fungi and seaweed. Glucan's role as a biologically active immunomodulator has been well documented for over 50 years. Initial interest in the immunomodulatory properties of polysaccharides was raised after experiments showing that a crude yeast cell preparation stimulated macrophages through activation of the complement system (Benacerraf and Sebestyen, 1957).

The best known effects of glucans consist of the augmentation of phagocytosis of professional phagocytes granulocytes, monocytes, macrophages and dendritic cells which direct stimulation of natural killer cells. In this regard, macrophages (Chihara et al., 1982; Vetvicka et al., 1996), considered to be the basic effector cells in host defense against bacteria, viruses, parasites and tumor cells, which play the most important role. There is evidence that glucan makes a considerable contribution toward the increased production of nitric oxide, one of the most effective reactive nitrogen species, by inducible nitric oxide synthase (iNOS) in macrophages (Ohno et al., 1996). Additional biological effects of glucans include stimulation of infectious immunity, activation of bone marrow cell production, anti-cancer effects and lowering of blood cholesterol (Kimura et al., 1994; Kogan, 2000; Vetvicka et al., 2009) for review see Novak and Vetvicka 2008).

Glucan is clearly not the only known immunomodulator in the entire world. Despite the fact that glucan, with over 10,000 published scientific papers, is the best studied and best documented natural modulator, other biologically active molecules exist. More and more manufacturers and retailers are experimenting with the preparation of various cocktails or mixtures of potentially bioactive powders. It is now very common to find glucan in combination with five or more ingredients, including Echinacea, Aloe vera, Astragalus and Goldenseal.

There are recent studies showing that some bioactive molecules have synergistic effects when combined with glucan. Numerous scientific studies have confirmed some beneficial effects when glucan was given in combination with vitamin C. The main reason why vitamin C shows synergistic effects is the fact that this vitamin has been proven to stimulate the exact same immune responses as glucan, that is, macrophage activities, natural killer cell activity and specific antibody formation. A mouse study revealed significant healing abilities of a glucan-vitamin C combination in the treatment of infection by *Mesocestoides corti*. The treatment resulted in positive modulation of liver fibrosis and pathophysiological changes (Ditteova et al., 2003).

Resveratrol (trans-3,4',5-trihydroxystilbene) is a non-flavonoid polyphenol found in various fruits and vegetables and is abundant in the skin of grapes. In addition to various biochemical, biological and

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pharmacological activities, resveratrol has been found to exhibit numerous immunomodulatory activities such as suppression of lymphocyte proliferation, changes in cell-mediated cytotoxicity, cytokine production (Gao et al., 2001) and induction of apoptosis (Losa, 2003).

Our study was based on a recent observation showing that seaweed-derived glucan elicited defense responses in grapevine and induced protection against *Botrytis cinerea* and *Plasmopara viticola* through the induction of production of two phytoalexins including resveratrol (Aziz et al., 2003). This led us to evaluate the possible synergetic effects of glucan and resveratrol on immune reactions.

MATERIALS 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.

Materials

Yeast-derived insoluble glucan #300 were purchased from Transfer Point (Columbia, SC). Resveratrol was purchased from Suan Farma, Paramus, NJ. Based on the HPLC analysis, it is 98.2% pure transresveratrol isolated from *Polygonum cuspidatum*. Anti-mouse CD4 antibodies conjugated with FITC were purchased from Biosource (Camarillo, CA).

Phagocytosis

The technique employing phagocytosis of synthetic polymeric micro spheres was described earlier (Vetvicka et al., 1982; 1988). Briefly: peripheral blood cells or isolated peritoneal cells were incubated *in vitro* with 0.05 ml of 2-hydroxyethyl methacrylate particles (HEMA; $5x10^8$/ml).

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

Flow cytometry

Cells were stained with monoclonal antibodies in 12 × 75 mm glass tubes using standard techniques for 30 min on ice. After washing with cold PBS, the cells were resuspended in PBS containing 1% BSA and 10 mM sodium azide. Flow cytometry was performed with a FACScan (Becton Dickinson, San Jose, CA) flow cytometer and the datas from over 10,000 cell sample were analyzed.

IL-2 production

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

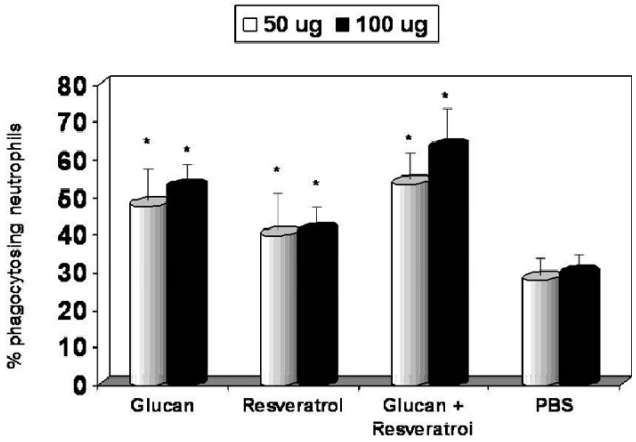

Figure 1. Effect of an ip. administration of glucan or resveratrol samples on phagocytosis by peripheral blood granulocytes. Each value represents the mean ± SD. *Represents significant differences between control (PBS) and tested samples at P ≤ 0.05 level. All experiments were performed in triplicates.

Antibody formation

Formation of antibodies was evaluated using ovalbumin as an antigen. Mice were injected twice (two weeks apart) with 100 ∝g of albumin and the serum was collected 7 days after last injection. Experimental groups were getting daily ip. injections of either glucan or resveratrol. Level of specific antibodies against ovalbumin was detected by ELISA. As positive control, combination of ovalbumin and Freund's adjuvant was used.

Statistics

Student's t-test was used to statistically analyse the data.

RESULTS

The effects of various glucans on macrophages are well established. However, in order to demonstrate that a new combination of immunomodulators really exhibits synergistic immunomodulatory characteristic, an evaluation of phagocytosis is necessary. We measured the effects of glucan or resveratrol on blood neutrophil phagocytosis of synthetic microparticles based on HEMA (Figure 1). Both glucan and resveratrol significantly stimulated the phagocytosis of synthetic particles. However, the combined preparation exhibited strong synergetic effect both on monocytes and neutrophils. Similar results were obtained when we measured the phagocytic ability of peritoneal macrophages *in vitro*. The effects of resveratrol alone

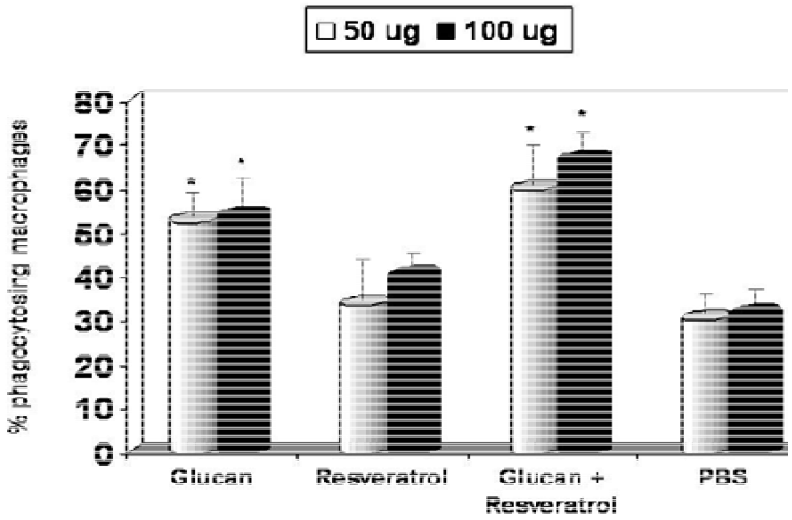

Figure 2. Effect of an ip. administration of glucan or resveratrol samples on phagocytosis by peritoneal macrophages. Each value represents the mean ± SD. *Represents significant differences between control (PBS) and tested samples at P ≤ 0.05 level. All experiments were performed in triplicates.

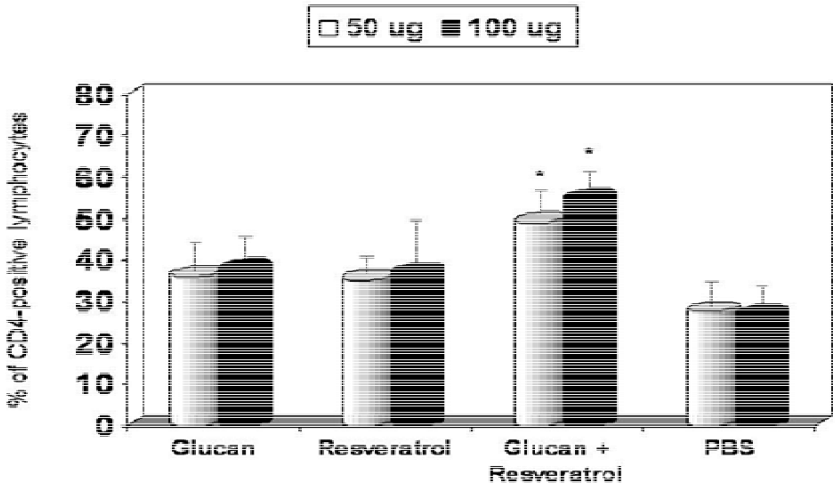

Figure 3. Effect of ip. injection of glucan or resveratrol on the expression of CD4 marker by spleen cells. The cells from three donors at each time interval were examined and the results given represent the means ± SD. *Represents significant differences between control (PBS) and samples at P ≤ 0.05 level. All experiments were performed in triplicates.

were less pronounced and not statistically significant, but again, there was strong statistically significant synergictic effect (Figure 2). Our preliminary experiments showed that these effects last up to 3 days after treatment (data not shown). These data were in agreement with previously published data using different types of glucan and resveratrol (Vetvicka and Yvin 2004; Vetvicka et al., 2007).

Next we evaluated the effects of the tested compounds on expression of CD4 marker on mouse splenocytes. Our data summarized in Figure 3 showed that, whereas both glucan and resveratrol increased the expression of this marker, their simultaneous effects were much stronger and statistically significant. No changes between lower (50 ∝g) and higher (100 ∝g) doses were observed. The cellular of peritoneal cavity was not influenced by either glucan or resveratrol (data not shown), so the observed changes were not caused by the influx of cells.

Evidence of the immunomodulating activity was also demonstrated through effects on the production of IL-2 by

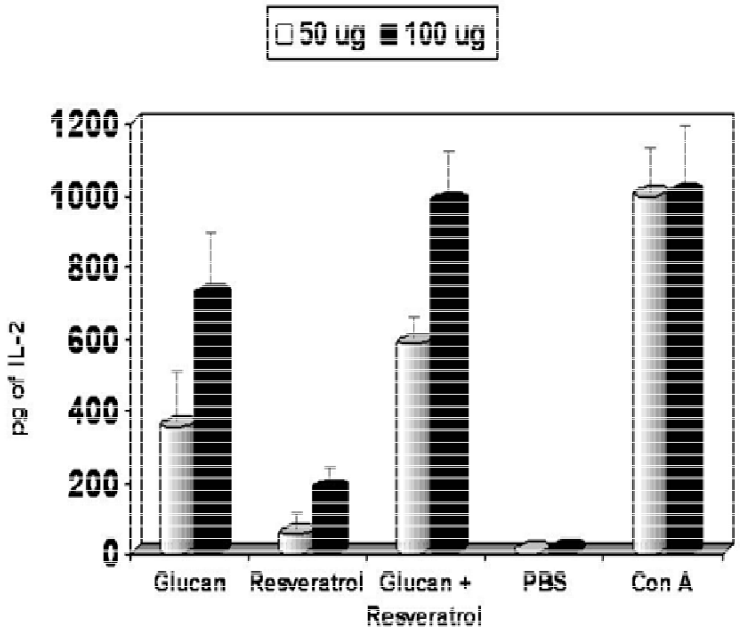

Figure 4. Effects of glucan or resveratrol on Con A-stimulated secretion of IL-2 by spleen cells. As the control (PBS) production of IL-2 is zero, all collumns represents significant differences between control (PBS) and samples at $P \leq 0.05$ level. All experiments were performed in triplicates.

spleen cells (Figure 4). The production of IL-2 was measured after 48 h *in vitro* incubation of spleen cells isolated from control and treated mice. Whereas glucan showed strong and dose-dependent stimulation of IL-2 secretion, resveratrol alone was only slightly effective and only in the high dose. However, when used together, they stimulated IL-2 secretion as much as Con A, serving as a positive control. As there was no IL-2 production with PBS alone, every increase shown in Figure 4 was statistically significant.

We then focused on the use of glucan and resveratrol as an adjuvant. As an experimental model, we used immunization with ovalbumin. Glucan and resveratrol combination were applied simultaneously with two intraperitoneal doses of antigen, a commonly used Freund's adjuvant was used as additional positive control. The results (Figure 5) showed that resveratrol had no effects. Glucan alone significantly elevated the antibody response to 200%, whereas a combination of glucan and resveratrol increased the antibody production four times. It must be noted, however, that none of the tested substances potentiated the humoral immunity to the level of Freund's adjuvant.

DISCUSSION

The immune system is a system of cells, organs and soluble molecules working in unison to defend the body against foreign pathogens. This system consists of numerous components, constantly on alert to find invading pathogens finding means to destroy them and eliminating them from our body. Individual cells interact with one another using physical contact or the secretion of various bioactive molecules. The innate system is considered to be the first line of defense and represents a significant part of the entire immune system. It includes mechanical barriers, cells such as macrophages and neutrophils and soluble factors such as the complement system and antimicrobial peptides. In many cases of infection, the innate immune mechanisms are sufficient to prevent full-blown infection.

The acquired immune system identifies the characterristic proteins of invading microorganisms and their toxins. This part of immunity consists of cells such as T and B lymphocytes and antigen-presenting cells (macrophages and dendritic cells). The lymphocytes recognize the invading pathogens by specific antibodies (B lymphocytes) or specific receptors (T lymphocytes). All types of immune cells work together symphoniously. They constantly interact with each other and function on the basis of information transferred using a complicated network of humoral factors such as enzymes and cytokines.

Immunomodulators usually offer systemic effects and details of the mechanisms of their effects are often unknown. This paper focused on hypothesis that glucan and resveratrol might together offer higher stimulation of

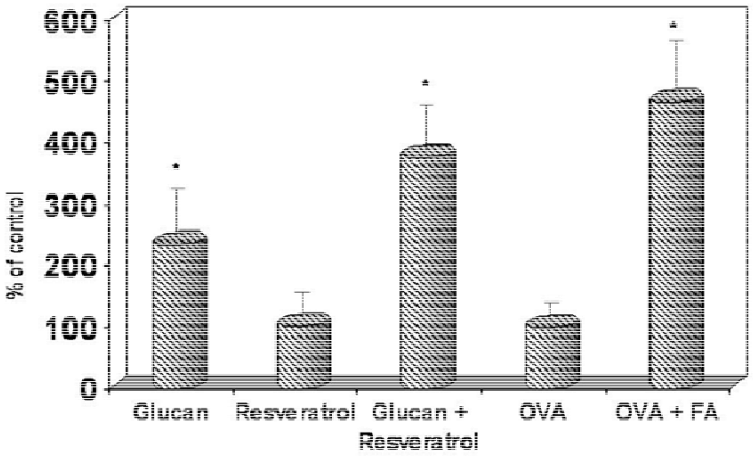

Figure 5. Effects of two ip. injections of glucan or resveratrol on formation of antibodies against ovalbumin. Mice were injected twice (two weeks apart) and the serum was collected 7 days after last injection. Level of specific antibodies against ovalbumin was detected by ELISA. As positive control, Freund's adjuvant was used. *Represents significant differences between control (ovalbumin alone) and samples at $P \leq 0.05$ level. All experiments were performed in triplicates.

immunity than individual molecules. Therefore we decided to monitor their effects on the most important reactions covering both branches of the immune reactions, that is, both cellular and humoral immunity.

Various types of immunomodulators, glucans in particular, are well established to stimulate phagocytosis (Abel et al., 1989). Therefore, the evaluation of this basic type of defense reaction is important for determining the effectiveness of any biologically active immunomodulator. We tested the peripheral blood leukocytes and peritoneal macrophages for changes in phagocytosis. Using synthetic microspheres based on 2-hydroxyethyl methacrylate, we found that both tested substances caused significant increase in phagocytosis, but the combined preparation showed significant synergetic effect on both macrophages and neutrophils. The data shown reflects the effects of a single injection of either glucan or resrevatrol.

Observations of the effects on expression of cell surface CD4 marker present on splenocytes demonstrated that the numbers of CD4 + lymphocytes were significantly affected. Again, the combined preparation of glucan and resveratrol showed synergetic effects. Two days after the application, the numbers returned to normal. A similar increase in the number of CD4-positive cells after glucan application has been described for lentinan (Arinaga et al., 1992) and Phycarine (Vetvicka and Yvin, 2004).

In addition to the direct effect on various cells of the immune system, the immunostimulating action of β-glucans is caused by potentiation of a synthesis and release of several cytokines such as TNFα, IFNγ, IL-1, and IL-2. This cytokine stimulating activity was found to be dependent on the triple helix conformation (Falch et al., 2000). It is hypothesized that glucans enhance leukocyte functions through increased cytokine secretion, particularly during early stages of infection. The potential effect of resveratrol on individual cytokines is much less clear. Some studies suggest that resveratrol can inhibit some IL-2 or TNF-α mediated functions (Lee and Moon, 2005; Kolgazi et al., 2006; Conover et al., 2006) represent only indirect proof of the resveratrol-cytokine interaction. Therefore, the evaluation of the potential systemic effect on IL-2 was particularly interesting. Using the previously established dose and time interval (Vetvicka and Yvin, 2004), we observed that resveratrol-induced only very low IL-2secretion. However, when used together with glucan, the synergetic effects were profound.

Glucans are usually considered modulators of the cellular branch of immune reaction and very little attention has been directed to their potential effects on antibody response. Regarding resveratrol, its effects on antibody response were never tested. We decided to take advantage of the recently published method of evaluating the use of glucan as adjuvant (Vetvicka and Vetvickova, 2007). Our results confirmed that glucan elevated the antibody response and that resveratrol alone has no activity. Surprisingly, simultaneous application of both agents showed very strong synergistic effects. Data presented in this paper represent further proof that combined preparations of glucan and resveratrol strongly stimulate both branches of immune reactions. A study attempting to reveal the exact mechanisms of these

effects is currently under progress.

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