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Glucan and Humic Acid: Synergistic Effects on the Immune System

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ABSTRACT Humic acids are compounds resulting from decomposition of organic matter. Despite their common presence, our knowledge of their biological effects is limited, and current findings are controversial. We decided to evaluate the immunological effects of two different types of humic acids, differing in source and biochemical characteristics. Using both components either alone or in combination with the well-established yeast-derived immunomodulator glucan, we measured their effects on both the cellular (phagocytosis and tumor suppression) and humoral (antibody production and cytokine secretion) branches of immune reactions. In summary, our results suggest that humic acids are biologically active immunodulators affecting both the humoral and cellular branches of immune reactions. In addition, the two humic acids studied here are working in synergy in stimulation of the immune reaction, supporting further studies of these natural immunomodulators.

KEY WORDS: • ***glucan*** • ***humic acids*** • ***immunity*** • ***phagocytosis***

INTRODUCTION

THE ROLE OF β1,3-GLUCAN as an immunomodulator has been well documented for over 50 years. The first interest in the immunomodulatory properties of polysaccharides was raised after experiments showing that a crude yeast cell preparation stimulated macrophages via activation of the complement system.[1] Further work identified the immunomodulatory active component as β1,3-glucan.[2] Numerous studies have subsequently shown that β1,3-glucans, either particulate or soluble, exhibit immunostimulating properties, including antibacterial and antitumor activities.[3,4] More than 2,500 publications have reported that β1,3-glucans, either soluble or particulate, exhibit significant immunomodulatory properties. Currently, glucans are considered to be one of the most efficient biological response modifiers (for review, see Novak and Vetvicka[5]).

Some studies suggested that bioactive molecules have synergistic effects when combined with glucan. Numerous reports have shown 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 shown to stimulate the exact same immune responses as glucan, *i.e.*, macrophage activities, natural killer cell activity, and specific antibody formation. A mouse study showed 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.[6] The same group found previously that yeast-derived glucan is a promising agent against several helminthic parasites.[7] With respect to the liver disease, schizophyllan glucan was shown to help against an ischemia-reperfusion injury of the liver. The mechanisms of these effects are probably due to the glucan-caused decrease of the expression of immediate early genes following injury to the liver.[8]

Humic substances occur mainly in heavily degraded peat. Humic acids (HAs) represent a group of high-molecular-weight macromolecules consisting of complex polymeric aromatic structures. Together with fulvic acids, they represent certain fractions of the group of organic compounds called humic substances, which are by some considered inert and by others biotoxic.[9] More detailed studies revealed controversial results: high doses of HA induced chromosomal abnormalities in intestinal cells[10] via oxidative DNA damage,[11] inhibited nuclear factor κB activation,[12] and stimulated the thymus[13] and neutrophils.[14] On the other hand, several interesting and potentially clinically important biological activities were recently associated with various types of HA, including antiviral properties[15] and proliferation of lymphocytes.[16] Also, addition of HA into the feed of cultured animals results in improved growth and health.[17]

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The recent observation showing that glucan's biological activities were significantly improved by resveratrol[18] led us to evaluate the possible synergetic effects of glucan and HAs on immune reactions.

MATERIALS AND METHODS

Animals

Female, 6–10-week-old BALB/c mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). All animal work was done according to the protocol of the Institutional Animal Care and Use Committee of the University of Louisville, Louisville, KY, USA. Animals were sacrificed by CO_2 asphyxiation.

Materials

RPMI 1640 medium, sodium citrate, ovalbumin, antibiotics, Wright's stain, *Limulus* lysate test E-TOXATE, Freund's adjuvant, and concanavalin A (Con A) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Fetal calf serum was from Hyclone Laboratories (Logan, UT, USA).

β1,3-Glucans

The glucans used in this study were purchased from the following companies: yeast-derived insoluble glucan number 300 from Transfer Point (Columbia, SC, USA) and soluble glucan laminarin from Sigma.

Extraction procedure

Two HAs were used in the experiments: one extracted from leonardite obtained from Czech Republic (HZ) and another one extracted from lignite obtained from China (HC). The different HAs were isolated, purified following the International Humic Substances Society procedure,[1,2] and freeze-dried.

Solid-state ^{13}C-nuclear magnetic resonance (NMR) spectroscopy

Solid-state ^{13}C-NMR spectra were obtained on a Bruker (Billerica, MA, USA) Avance AV-400WB (9.4-T) spectrometer at 100.47 MHz using the cross-polarization magic angle spinning technique, with a spinning speed of 12 kHz, 90° pulse width, 30 msecond acquisition time, and 4.0 second delay.

Elemental analysis

The carbon, hydrogen, and nitrogen contents of the lyophilized samples were analyzed in duplicate by a LECO® (St. Joseph, MI, USA) CHN 900 analyzer. The oxygen content was determined by difference (ash-free basis).

Phagocytosis in vitro

Phagocytosis was measured *in vitro* using synthetic microspheres (2-hydroxyethyl methacrylate [HEMA] particles) after intraperitoneal injection of glucans and/or HA as described earlier.[19,20] In brief, blood or isolated peritoneal cells were incubated with 0.05 mL of HEMA particles (5×10^8/mL). The test tubes were incubated at 37°C for 60 minutes, with intermittent shaking. Smears were stained with Wright's stain. Cells with three or more HEMA particles were considered positive. The same smears were also used for evaluation of cell types.

Evaluation of interleukin (IL)-2 production

Purified spleen cells (2×10^6/mL in RPMI 1640 medium with 5% fetal calf serum) were added into wells of a 24-well tissue culture plate. After addition of 1 μg of Con A into positive control wells, cells were incubated for 72 hours in a humidified incubator (37°C, 5% CO_2). At the end point of the incubation, supernatants were collected, filtered (pore size, 0.45 μm), and tested for the presence of IL-2. IL-2 levels were measured using a Quantikine® mouse IL-2 kit (R&D Systems, Minneapolis, MN, USA).

Cytokine array

Individual cytokines were measured in mouse serum by Allied Biotech (Ijamsville, MD, USA). Mice were injected with the test combination, 24 hours later the mice were sacrificed, and serum was collected and stored in a −80°C freezer. For the cytokine analysis, we used the protein microarray services provided by Allied Biotech. In brief, the services used a sandwich antibody-based protein detection multiplex assay. A streptavidin-Cy5 conjugate was used for assay detection. The assay was done in quadruplicate with positive and negative controls spotted on each microarray. The assay detects the following cytokines: IL-2, interferon-γ, tumor necrosis factor (TNF)-α, IL-8, IL-12 p70, IL-12 p40, IL-4, IL-6, IL-10, IL-5, interferon-inducible protein-10, macrophage inflammatory protein-1β, IL-13, IL-1β, and monocyte chemotactic protein (MCP).

Tumor inhibition in vivo

Mice were injected directly into the mammary fat pads with 1×10^6 Ptas64 cells in phosphate-buffered saline (PBS) per mouse. 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. After treatment, the mice were sacrificed, and tumors were removed and weighed.

Antibody formation

Mice were injected twice (2 weeks apart) with 100 μg of ovalbumin, and serum was collected 7 days after the last injection. Levels of specific antibodies against ovalbumin were detected by enzyme-linked immunosorbent assay. As the positive control, Freund's adjuvant was used.

Statistics

Student's *t* test was used to statistically analyze the data.

RESULTS

As can be observed in the elemental analysis and particularly in the ^{13}C-NMR analysis, HZ presents more functionality than HC in both the aromatic and aliphatic moieties. This functionality is expressed in higher contents of phenolic, carbonylic carbon, and *O*-alkyl C. However, HC presents a marked aromatic character, whereas HZ presents a predominant aromatic character with significant aliphatic arrangements. Thus, we have two HAs with very different structures: HZ is more *O*-functionalized and with combining aromatic and aliphatic moieties, and HC is less functionalized but has high aromatic character (Tables 1 and 2).

First we measured the effects of HA with and without glucan on numbers of cells in the peritoneal cavity. Using the same combinations as in all subsequent experiments, we observe no changes in cellularity (with respect to both total numbers and differential counts) after either intraperitoneal or oral application (data not shown). In all cases, glucan, HA, or both were dissolved in PBS, which was also used as a negative control.

The effects of various glucans on macrophages are well established. However, in order to demonstrate that a new combination of immunomodulators really exhibits an immunomodulatory property, an evaluation of phagocytosis is necessary. First, we measured the effects of glucan and/or HA on *in vitro* phagocytosis of synthetic HEMA microspheres in peripheral blood (Fig. 1). Both glucan and HA stimulated the internalization of synthetic particles, but the combined preparation, particularly in the 1:1 ratio, exhibited a significant synergetic effect on blood neutrophils. Identical results were achieved when we measured *in vitro* phagocytosis of peritoneal macrophages (Fig. 2). In both cases, the cells were isolated from mice injected with glucan and/or HA.

Evidence of the immunomodulating activity was also demonstrated through effects on the production of IL-2 by spleen cells (Fig. 3). The production of IL-2 was measured

TABLE 1. ELEMENTARY ANALYSIS OF HA SAMPLES

HA	*% C*	*% H*	*% N*	*% O*[a]
HZ	48.2	2.99	0.98	48.9
HC	57.5	1.60	1.10	39.8

[a]By difference.

TABLE 2. ^{13}C-NMR SPECTROSCOPY OF HA SAMPLES

	Region (ppm)				
HA	*Alkyl C (0–45)*	*O-Alkyl C (45–110)*	*Aromatic C (110–160)*	*Phenolic C (140–160)*	*Carbonylic C (160–215)*
HZ	27.4	12.6	46.5	12.8	13.5
HC	13.8	3.2	79.5	5.4	3.5

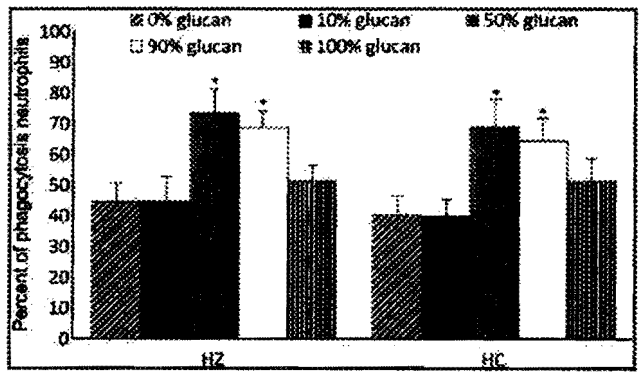

FIG. 1. Potentiation of *in vitro* phagocytosis of synthetic microspheres (HEMA particles) by intraperitoneally injected glucans and/or HAs. A total of 100 μg of material in various ratios was injected. Control values (PBS only) were 33.4%. Peripheral blood neutrophils with three and more HEMA particles were considered positive. Data are mean $\pm$ SD values from three separate experiments (five mice per experimental group). *Significant differences at the $P \leq .05$ level.

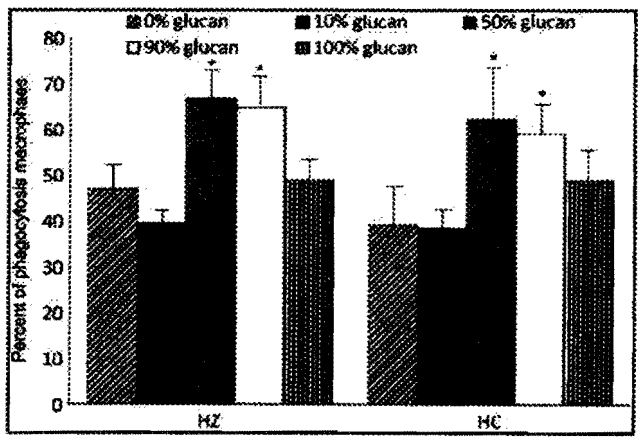

FIG. 2. Potentiation of *in vitro* phagocytosis of synthetic microspheres (HEMA particles) by intraperitoneally injected glucans and/or HA. A total of 100 μg of material in various ratios was injected. Control values (PBS only) were 36.5%. Peritoneal macrophages with three and more HEMA particles were considered positive. Data are mean $\pm$ SD values from three separate experiments (five mice per experimental group). *Significant differences at the $P \leq .05$ level.

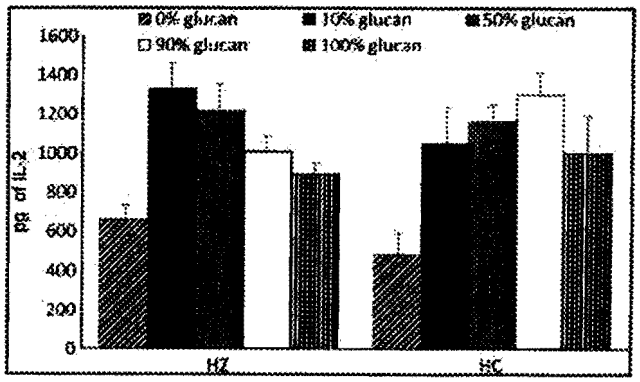

FIG. 3. Effects of glucan–HA combinations on Con A-stimulated secretion of IL-2 by spleen cells. As the secretion of IL-2 by nonstimulated splenocytes (PBS group) is zero, even the lowest stimulation was statistically significant. Data are mean $\pm$ SD values from three separate experiments (five mice per experimental group).

TABLE 3. EFFECT OF HA SAMPLES AND GLUCAN ON CYTOKINE SECRETION

HA	*Glucan*	*IL-2*	*IL-4*	*IL-5*	*IL-6*	*TNF-α*	*MCP-1*
HZ							
100%	0%	111.2 ± 8.8	149.2 ± 11.8	77.1 ± 4.4	56.2 ± 2.3	109.1 ± 5.5	1.0 ± 0.1
90%	10%	233.8 ± 11.9	155.6 ± 8.8	56.6 ± 2.2	50.5 ± 4.5	120.1 ± 6.7	8.1 ± 1.1
50%	50%	534.6 ± 21.2	651.5 ± 21.8	268.8 ± 11.5*	109.2 ± 3.3*	434.4 ± 21.7*	17.2 ± 1.1*
10%	90%	599.9 ± 34.5	601.2 ± 33.8	301.6 ± 22.0*	88.8 ± 4.4*	443.4 ± 19.9*	9.2 ± 0.9
0%	100%	597.2 ± 34.8	580.9 ± 40.1	119.6 ± 18.1	48.2 ± 3.8	270.1 ± 9.9	6.0 ± 1.1
HC							
100%	0%	36.6 ± 1.8	97.1 ± 4.4	21.2 ± 1.9	61.5 ± 3.5	76.5 ± 3.9	0
90%	10%	148.4 ± 8.8	142.2 ± 5.5	26.1 ± 2.2	38.4 ± 4.1	134.4 ± 7.1	1.0 ± 0.1
50%	50%	465.4 ± 22.3	712.5 ± 34.9	199.7 ± 9.1	101.1 ± 4.5	354.5 ± 18.7	9.1 ± 1.7*
10%	90%	715.2 ± 42.9	608.2 ± 36.5	215.3 ± 11.1*	188.4 ± 9.9*	489.6 ± 17.9*	4.4 ± 0.8

Data are mean ± SD values from three separate experiments (five mice per experimental group). Negative controls (PBS only) were always zero.
*Represents significant differences between glucan–HA and either glucan only of HA-only groups at the $P \leq .05$ level.

after a 72-hour *in vitro* incubation of spleen cells isolated from control and treated mice. Again, treatment of mice with a combination of glucan and HA resulted in significant stimulation of IL-2 production. In the case of HZ, the highest stimulation was found with a 9:1 HZ-glucan ratio. In the case of HC, the highest production of IL-2 was found in the 1:9 ratio. All combinations showed much stronger stimulation that either glucan or HA alone. As the secretion of IL-2 by nonstimulated splenocytes (PBS group) is zero, even the lowest stimulation was statistically significant.

After the initial experiments, we measured the secretion of cytokines from mice injected intraperitoneally with the test material 24 hours earlier. The results shown in Table 3 clearly demonstrate several important observations: both HA and glucan stimulated the secretion of only six out of the 14 tested cytokines (IL-2, IL-4, IL-5, IL-6, TNF-α, and MPC-1), with the only exception being HC, which did not stimulate production of MCP-1. With respect to HZ, combination with glucan resulted in higher stimulation of IL-5, IL-6, and TNF-α secretion than in the case of HZ or glucan alone. A slightly different situation was found with the HC–glucan combination, for which elevated levels of IL-5, IL-6, and TNF-α were found only in the 1:9 ratio. In both cases we found only a small stimulation of MCP-1 secretion.

We then focused on the use of our combinations as an adjuvant. As an experimental model, we used immunization with ovalbumin. Glucan, HA, or both were applied together with two intraperitoneal doses of antigen; the commonly used Freund's adjuvant was used as an additional positive control. The results (Fig. 4) showed that the tested combination exhibited in all cases significant adjuvant activity against antigen alone (optical density, 0.311 ± 0.026). In the case of HZ, the highest stimulation was achieved with the 9:1 ratio, whereas in the case of HC we found the highest stimulation with the 1:1 and 1:9 ratios. It must be noted, however, that none of the glucans potentiated the humoral immunity to the level of Freund's adjuvant (optical density, 1.67 ± 0.22).

In the final step, mice challenged with Ptas64 mammary tumors were tested for a therapeutic response to daily intraperitoneal injections of the tested substances (Fig. 5). This experiment was repeated three times (three mice per experimental group) with similar results. Control values (PBS only) showed mean tumor weight of 699.7 ± 38.5 mg. Our data showed that in the case of HZ, the molecule responsible for the strong inhibition of tumor growth is clearly the glucan, as glucan-caused inhibition was the same as with HZ–glucan inhibition. Regarding the HC–glucan combination, the 1:1 ratio showed the strongest inhibition of cancer growth from all tested samples. In both cases, HA alone showed no significant activity.

DISCUSSION

Humic substances are the main components of humus in the soil. They are produced by chemical and microbial degradation of organic matter coming from plants and ani-

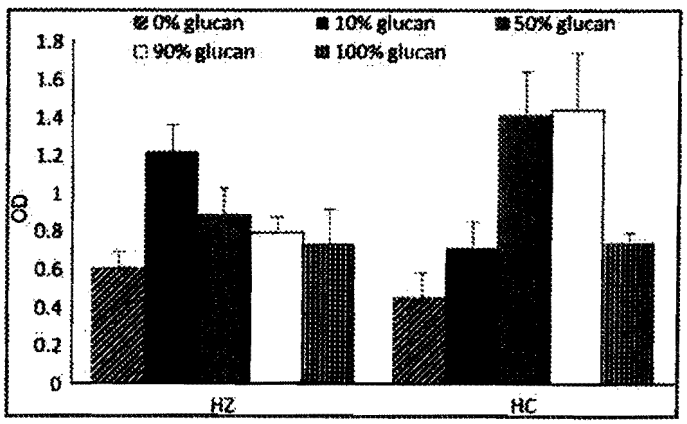

FIG. 4. Effects of two intraperitoneal injections of tested HA–glucan combinations on formation of antibodies against ovalbumin. Mice were injected twice (2 weeks apart), and serum was collected 7 days after last injection. The level of specific antibodies against ovalbumin was detected by enzyme-linked immunosorbent assay. As the positive control, Freund's adjuvant was used. Data are mean ± SD values from three separate experiments (five mice per experimental group). *Significant differences between control (ovalbumin alone) and samples at the $P \leq .05$ level. OD, optical density.

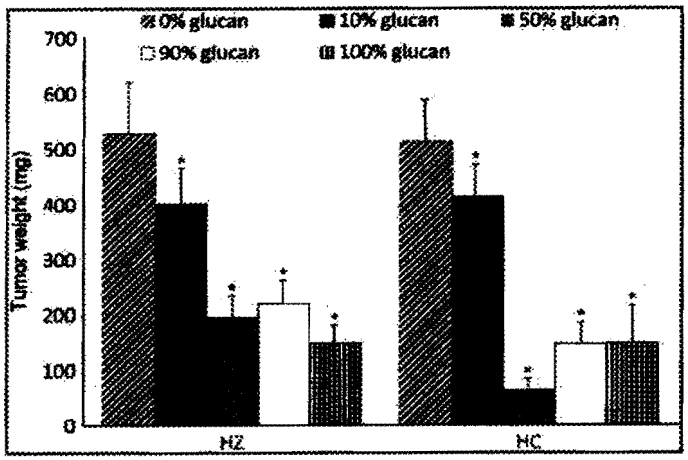

FIG. 5. HA–glucan therapy of BALB/c mice with Ptas64 mammary carcinoma. Data from three independent experiments are shown (three mice per experimental group). For each experiment, groups of mice were tested for a response to a therapy as indicated by the weight of tumors after 2 weeks of therapy. For each experiment, individual groups were given daily intraperitoneal injections of 100 μg of HA, glucan, or the combination. The control group of mice given daily intraperitoneal injections of PBS had a tumor weight of 699.7 ± 38.5 mg. Data are mean $\pm$ SD values. *Significant differences between treated and untreated groups at the $P \leq .05$ level.

mals. As a function of their solubility as a function of pH, three main organic humus fractions can be differentiated: HA, which is soluble at alkaline pH but insoluble at acid pH; fulvic acid, which is soluble at any pH; and humane, which is insoluble at any pH. The structure of these substances is not clear. From a qualitative point of view, they present the same type of functional groups and structural arrangements. However, the polydisperse nature of the systems makes it difficult to suggest definitive structures. In general, they are acid polyelectrolytes with different molecular weights and sizes and with diverse aromatic character.[21] A special feature of these substances is their ability to affect the metabolism and development of different organisms. Thus, effects of humic substances on plant metabolism, microbial activity, and animal development have been described in many other studies.[22–24] However, studies relating structural features and biological activity are scarce. Different studies have shown that a practical way to characterize humic substances is the complementary use of ^{13}C-NMR and elemental analysis.[24] We have applied these techniques to the two HAs used in these experiments. As can be observed in the elemental analysis and particularly in the ^{13}C-NMR analysis, HZ presents more functionality than HC in both the aromatic and aliphatic moieties. This functionality is expressed in higher contents of phenolic, carbonylic carbon, and *O*-alkyl C. However, HC presents a marked aromatic character, whereas HZ presents a predominant aromatic character with significant aliphatic arrangements. Thus, we have two HAs with very different structures: HZ is more *O*-functionalized and with combining aromatic and aliphatic moieties, and HC is less functionalized but with high aromatic character (Tables 1 and 2).

Various types of immunomodulators, glucans in particular, are well known to stimulate phagocytosis.[25,26] Therefore the evaluation of this basic type of immune reaction is important for determination of the effectiveness of any biologically active immunomodulator. We tested peripheral blood leukocytes and peritoneal macrophages for changes in phagocytosis using synthetic microspheres based on HEMA. These microparticles have a slight negative charge and therefore do not specifically adhere to the cell surface, which guarantees that only actively phagocytosing cells will internalize these inert particles.[27] This fact was verified by phase-contrast microscopy, electron microscopy, and quenching. We found that both substances tested caused significant increases in phagocytosis *in vitro*, but the combined preparation showed a significant synergetic effect on both macrophages and neutrophils. The data shown reflect the effects of a single injection of test substances, but our additional experiments found that similar effects can be observed after oral administration (data not shown). Stimulation observed in these experiments resulted from direct cell activation, as the numbers and percentages of cell types in blood or the peritoneal cavity did not change.

In addition to the direct effect on various cells of the immune system, the immunostimulating action of β-glucans and other immunomodulators is caused by potentiation of synthesis and release of several cytokines. First we focused on the stimulation of IL-2 production by spleen cells *in vitro* and found that HA–glucan combinations stimulated not only higher release of IL-2 than the tested substances alone, but in the case of 90% HZ with glucan release was even higher than with Con A.

Later we evaluated the effects on a panel of 14 different cytokines in serum. Depending on the HC–glucan ratio, the stimulation was more pronounced with HZ than with HC. However, in all cases the production of cytokines was higher than with HA alone. The most stimulated cytokines were IL-5, IL-6, and TNF-α; eight of the total of 14 tested cytokines were not detected.

Cytokines are important intercellular communicators, and their crucial role in information flow between different parts of immune system is well established. Reports on the role of ILs in cancer reveal both cancer growth-inhibitory functions and cancer growth-promoting properties.[28] The nature of malignant progression is complex, and cytokines produced by malignant cells can function as both autocrine growth factors and immunomodulators.[28]

As some recent studies established that glucans can also support the humoral branch of the immune reaction by serving as an adjuvant,[29] we compared the adjuvant activities of the tested glucan–HA combinations with that of Freund's adjuvant. Our results showed that even though the activities were always lower than those of Freund's adjuvant, they were nevertheless significant, with the highest activity found with two combinations of glucan–HC.

Finally, we decided to test the possible effects of HA–glucan combinations on *in vivo* growth of the mouse breast tumor cell line Ptas64. Our previous work demonstrated that there is a high similarity of mouse and human CR3 in

response to glucans, which makes the mouse tumor models suitable for investigation of glucans.[30] We used the same experimental design as published before using yeast-derived glucan.[31] Similar to the antibody response, the highest effects were found with a 1:1 ratio of HC–glucan, where we observed over 92% inhibition. This result indicates that aromaticity could play an important role in this antitumoral effect of HAs because HZ, which presents a significantly lower aromaticity than HC, did not show this activity.

Lipopolysaccharide contamination might mask the real effects of any immunomodulator. Therefore we checked the lipopolysaccharide contamination of our solutions. Observed values were always lower than 10 EU. In addition, we functionally depleted lipopolysaccharide from both glucan and HA by treatment with 10 μg/mL polymixin B. We found identical results in all cases. The similarity between results obtained with regular and lipopolysaccharide-free material indicated that the minor lipopolysaccharide presence is not responsible for elevation of immunological activities and/or the antitumor response.

To summarize our data, our report suggests that HAs are biologically active immunodulators affecting both the humoral and cellular branches of immune reactions. In addition, both HZ and HC are working in synergy in stimulation of the immune reaction, a finding supporting further studies of these natural immunomodulators.

AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

REFERENCES

1. Benacerraf B, Sebestyen MM: Effect of bacterial endotoxins on the reticuloendothelial system. *Fed Proc* 1957;16:860–867.
2. Rigi SJ, Di Luzio NR: Identification of a reticuloendothelial stimulating agent in zymosan. *Am J Physiol* 1961;200:297–300.
3. Di Luzio NR, Williams DL, McNamee RB, Edwards BF, Kitahama A: Comparative tumor-inhibitory and anti-bacterial activity of soluble and particulate glucan. *Int J Cancer* 1979;24:773–779.
4. Mimura H, Ohno N, Suzuki I, Yadomae T: Purification, antitumor activity, and structural characterization of β-1,3-glucan from *Peziza vesiculosa*. *Chem Pharm Bull* 1985;33:5096–5099.
5. Novak M, Vetvicka V: Glucans as biological response modifiers. *Endocr Metab Immune Disord* 2009;9:67–75.
6. Ditteova G, Velebny S, Hrckova G: Modulation of liver fibrosis and pathological changes in mice infected with *Mesocestoides corti* (*M. vogae*) after administration of glucan and liposomized glucan in combination with vitamin C. *J Helmintol* 2003;77:219–226.
7. Velebny S, Tomasovicova O, Hrckova G, Dubinsky P: *Toxocara canis* in mice: are liposomes and immunomodulator able to enhance the larvicidal effect of the anthelminthic? *Helminthologia* 1997;34:147–153.
8. Kukan M, Szatmary Z, Lutterova M, Kuba D, Vajdova K, Horecky J: Effects of sizofiran on endotoxin-enhanced cold ischemia-reperfusion injury of the rat liver. *Physiol Res* 2004;53: 431–437.
9. Bittner M, Janosek J, Hilscherova K, Giesy J, Holoubek I, Blaha L: Activation of Ag receptor by pure humic acids. *Environ Toxicol* 2006;21:338–342.
10. Bernacchi F, Ponzanelli I, Minunni M, Falezza A, Loprieno N, Barale R: *In vivo* cytogenetic effects of natural humic acid. *Mutagenesis* 1996;11:467–469.
11. Hseu JC, Chen SC, Chen YL, Chen JY, Lee ML, Lu FJ: Humic acid induced genotoxicity in human peripheral blood lymphocytes using comet and sister chromatid exchange assay. *J Hazard Mat* 2008;153:784–792.
12. Gau RJ, Yang HL, Chow SN, Suen JL, Lu FJ: Humic acid suppresses the LPS-induced expression of cell-surface adhesion proteins through the inhibition of NF-κB activation. *Toxicol Appl Pharmacol* 2000;166:59–67.
13. Madej JA, Kuryszko J, Garbulinski T: The influence of long-term administration of tolpa peat preparation on immune reactivity in mice. I. Morphological changes in thymus. *Acta Poloniae Pharmaceut* 1993;50:397–404.
14. Chen CH, Liu JJ, Lu FJ, Yang ML, Lee Y, Huang TS: The effect of humic acid on the adhesibility of neutrophils. *Thromb Res* 2003;108:67–76.
15. Klocking R, Sprossig M, Witzler P, Thiel KD, Helbig B: Antiviral wirkssame huminsaureahnliche Polymere. *Z Physiother* 1983;33:95–101.
16. Joone GK, Dekker J, Jansen van Rensburg CE: Investigation of the immunostimulatory properties of oxihumate. *Z Naturforsch C* 2003;58:263–267.
17. Islam KMS, Schuhmacher A, Gropp JM: Humic acid substances in animal agriculture. *Pakistan J Nutr* 2005;4:126–134.
18. Vetvicka V, Volny T, Saraswat-Ohri S, Vashishta A, Vancikova Z, Vetvickova J: Glucan and resveratrol complex—possible synergetic effects on immune system. *Biomed Pap Med Fac* 2007;151:41–46.
19. Vetvicka V, Fornusek L, Kopecek J, Kaminkova J, Kasparek L, Vranova M: Phagocytosis of human blood leukocytes: a simple micromethod. *Immunol Lett* 1982;5:97–100.
20. Vetvicka V, Holub M, Kováru H, Siman P, Kováru F: Alpha-fetoprotein and phagocytosis in athymic nude mice. *Immunol Lett* 1988;19:95–98.
21. Stevenson FJ: *Humus Chemistry, Genesis, Composition, Reactions*. Wiley Intersciences, New York, 1982,
22. Chen Y, Aviad T: Effects of humic substances on plant growth. In: *Humic Substances in Soil and Crop Sciences: Selected Readings* (MacCarthy P, Clapp CE, Malcolm RL, Bloom PR, eds.). American Society of Agronomy, Madison, WI, 1990, pp. 161–186.
23. Piccolo A: Humus and soil conservation. In: *Humic Substances in Terrestrial Ecosystems* (Piccolo A, ed.). Elsevier, Amsterdam, 1996, pp. 225–264.
24. Senesi N, Miano TM, eds.: *Substances in the Global Environment and Implications on Human Health*. Elsevier, Amsterdam, 1994.
25. Abel G, Szollosi J, Chihara G, Fachet J: Effect of lentinan and mannan on phagocytosis of fluorescent latex microbeads by mouse peritoneal macrophages: a flow cytometric study. *Int J Immunopharmacol* 1989;11:615–621.
26. Tomoda M, Ohara N, Shimizu N, Gonda R: Characterization of a novel glucan, which exhibits reticuloendothelial system-potentiating and anti-complementary activities, from the rhizome of *Cnidium officinale*. *Chem Pharm Bull (Tokyo)* 1994;42: 630–633.
27. Vetvicka V, Fornusek L: Polymer microbeads in immunology. *Biomaterials* 1987;8:341–345.

28. Mocellin S, Wang E, Marincola FM: Cytokines and immune response in the tumor microenvironment. *J Immunother* 2001;24: 392–407.
29. Vetvicka V, Vetvickova J: A comparison of injected and orally administered β-glucans. *J Am Nutr Assoc* 2008;11:42–49.
30. Xia Y, Vetvicka V, Yan J, Hanikyrova M, Mayadas TN, Ross GD: The β-glucan-binding lectin site of mouse CD3 (CD11b/CD18) and its function in generating a primed state of the receptor that mediates cytotoxic activation in response to iC3b-opsonized targets. *J Immunol* 1999;162:2281–2290.
31. Yan J, Vetvicka V, Xia Y, Coxon A, Carroll MC: β-Glucan, a "specific" biologic response modifier that uses antibodies to target tumors for cytotoxic recognition by leukocyte complement receptor type 3 (CD11b/CD18). *J Immunol* 1999;163:3045–3052.