Release of sFasL by Monocytes and Lymphocytes Triggered by Betaglucan and Zymosan

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ABSTRACT

Background: Polysaccharides have long been used as immune-modulators in various pathologic conditions including inflammation and solid malignancies. **Objective:** To evaluate the effects of Zymosan and Betaglucan on cytotoxic reactions in an effector-target conjugate system. **Methods:** Blood was obtained from 20 healthy subjects; purified mononuclear leukocytes (monocytes and lymphocytes) were extracted and cultured as effector cells by a cytotoxic method. Both adherent and non-adherent cells interacted with the K562 myeloid cell line. The effector-target (E:T) ratio was 1:1, 1:10, and 1:20. To evaluate stimulatory effects of Betaglucan and Zymosan on cytotoxic reactions, samples were divided into case and control groups based on the presence or absence of Betaglucan and Zymosan. MTT assay and sFas ligand (sFasL) concentrations were used to assess the increased killing capacity of effector cells.
Results: Our results revealed that Zymosan and Betaglucan can induce cytotoxic responses in macrophages and lymphocytes ($P<0.05$). The best result was achieved with E:T ratio of 1:1. Both macrophages and lymphocytes produced sFasL following stimulation by Zymosan and Betaglucan, however, the level of production was not statistically significant ($P>0.05$).
Conclusion: Zymosan and Betaglucan can be used as enhancers of the killing capacity of the immune cells; therefore, Betaglucan and Zymosan can be applied as systemic stimulators of the immune response in inflammation and chronic infection.

Keywords: Effector-target Conjugate, Cytotoxic Reactions, Macrophages, Lymphocytes, Zymosan, Betaglucan

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INTRODUCTION

Zymosan is basically a crude and primitive version of Betaglucan, derived from the cell wall of Sacharomyces Cervisiae (baker's yeast) (1-3). Both Zymosan and Betaglucan are biological response modifiers that regulate natural host immune responses against most pathogens and also play a major role in the immune response against malignances, especially Betaglucan which promotes natural killing mechanisms by activating macrophages and natural killer cells (NK cells) (4-8). In addition to specific stimulation of cell surface receptors, Betaglucan also non-specifically activates the immune system by enhancing the release of biologically important molecules such as TNF-α, IL 1, IL 6, hydrogen peroxide, and IFN γ (9-11).

Several studies have focused on the role of Betaglucan as a stimulator of immune defense mechanisms against solid tumors, especially breast cancer and sarcoma, in which NK cell activation has a major role (12). The aim of this study was to establish the actual mechanisms determining the killing capacity of effector cells including monocyte derived macrophages (MDMs) and lymphocytes against suspended tumor cells.

MATERIALS AND METHODS

Tumor Cell Line Preparation. K562 cell line, a human myeloid leukemia cell line, was suspended in the RPMI-1640 medium supplemented with 10% (V/V) FBS (Gibco), penicillin, streptomycin, and fungizon.

Effector Cell Preparation. Effector cells used in the cytotoxicity assay were peripheral blood mononuclear cells (PBMN) from 20 healthy male volunteers (20-30 years old) isolated by Ficoll–Hypaque centrifugation of venous blood. PBMNs were incubated in a plastic flat-bottom multi-well (96 well) tissue culture plate for 2 hours at 37°C. The adherent cells (macrophages) and non-adherent cells (lymphocytes) were separated and harvested as effector cells. For effector cell activation, preliminary PBMNs were incubated in the presence of Zymosan and Betaglucan.

Preparation of Zymosan and Betaglucan Particles. Zymosan (Sigma) was boiled for 30 minutes in 0.15 M of NaCl, then washed and re-suspended in 0.15 M NaCl. The final number of particles was 6×10^7/mg, quantified by nebular slide. Glucan particles (Sigma) were re-suspended in saline and sonicated for 1 minute, using a micro-tip ultrasonic disrupter probe. One milligram of glucan contains about 1.01×10^{10} particles determined by a nebular slide, according to the method described by Keeling et al and Janusz et al (6,7).

Tissue Culture Medium. RPMI-1640 supplemented with 10% (V/V) FBS, 2 µm glutamine,100µg/mL penicillin, and 100µg/mL streptomycin (Gibco) was used for cell culture and incubation. Cultures were maintained at 37°C with 5% CO_2 humidified atmosphere. Adherent cells were stained with non-specific esterase (NSE) in which up to 85% of cell preparation was positive.

PBMC Stimulation. PBMNs were categorized in 3 groups in separate wells, two test groups and one control group. Each group was further subdivided into two groups, based on the type of effector cell (MDMs or lymphocytes). In test groups, Zymosan (40×10^6/well) and Betaglucan (15×10^6/well) were added to each well prior to effector-target conjugation. Various numbers of effector cells (2×10^5/well, 1×10^5/well,

and 1×10^4/well) were conjugated with 1×10^4/well of K562 target cells to have effector-target (E:T) ratios of 1:1, 1:10, and 1:20, respectively. According to the method described by Brousseau et al and Fernandez et al (13,14). In the control group, effectors were conjugated with target cells without addition of Zymosan and Betaglucan.

M.T.T Assay. 1×10^4/well of K562 cells were plated in 96 flat-bottom well plates (Linbro). After conjugation, plates were centrifuged at 65g for 5 minutes and incubated at 37˚C with 5% CO_2 for 24 hours. Quintuplicate wells were used for different E:T ratios. Following incubation and two consecutive washings with fresh medium, 50µL of M.T.T solution (Sigma/St. Louis MO, 2mg/mL) was added to each well followed by 150µL of complete medium and incubated at 37˚C for four hours. To dissolve the formasan crystals formed by viable cells, 100µL acidic isopropanol solution was directly added to each plate. Plates were shaken for 10 minutes at room temperature until all crystals were dissolved. The absorbance at a wavelength of 540nm on a scanning multi-well spectrophotometer (ELISA reader) was immediately measured. The mean optical density of each quintuplicate sample was calculated (15,16).

Fas Ligand Assay. A commercial ELISA method was used for quantitative measurement of human soluble fas ligand (sFasL), using biotin-conjugated streptavidin HRP kit (BMS160/2).

Statistical Analysis. Paired data obtained from M.T.T assay were analyzed by a single rank test. Will Coxon's test was applied to determine the significance of nonspecific cytotoxic effects seen by addition of Zymosan and Betaglucan on the effector-target systems. The differences between sFasL values were analyzed using ANOVA test. P-values less than 0.05 were considered significant.

RESULTS

To assess Zymosan and Betaglucan stimulatory affects on cytotoxic reactions against target cells, two different methods were used. In MTT assay, the effector cells of control group, demonstrated a significant increased activity when cultured with target cells (MDMs: $P<0.006$, lymphocyte: $P<0.014$).

As the number of effector cells increased, the magnitude of cytotoxic reactions was increased too ($P<0.001$, $P=0.00$, and $P<0.006$, respectively). In the case groups lymphocytes were stimulated more profoundly compared with the control group (without Zymosan), the best result was observed with lymphocyte concentration of 2×10^5cell/well ($P<0.048$), in contrast it had a negative effect on target cells alone (Table 1). Increasing the concentration of effector cells in the cytotoxic system in the presence of Zymosan had no distinguishable effect on cytotoxic reactions.

Betaglucan in the absence of target cells had a stimulatory effect on effector cells especially MDMs, however, in the effector-target conjugate system Betaglucan did not evoke cytotoxic reactions in spite of inducing cell production in different effector-target ratios ($P<0.180$, $P<0.255$, and $P<0.287$). Betaglucan also had a slight positive effect on the cytotoxic capacity of lymphocytes. This effect was seen in 3 wells with different concentrations of lymphocytes ($P<0.007$, $P<0.019$, and $P<0.003$). Betaglucan had a positive effect on MDMs and lymphocytes and at the same time, it improved the proliferation rate and metabolic activity of target cells.

Fas Ligand Assay. Lymphocytes and MDMs showed increased secretion of sFasL following stimulation. This increase in sFasL secretion was not affected by the type of effector cell or the stimulants used (P>0.05). In the cytotoxic system, sFasL was only detectable in supernatants of culture media with the concentration of 2×10^5 cells/well. (Table 2)

Table 1. The comparison of mean ± SD of optical density measurements for M.T.T assay between the three studied groups P<0.911, P<0.341, and P<0.117 for Zymosan and P<0.0287, P<0.255, and P<0.182 for Betaglucan

Test & Control groups	Zymosan (Mean ± SD)					Betaglucan (Mean ± SD)					Control (Mean ± SD)				
	Effector	Effector + Target	Effector	Effector + Target	Target	Effector	Effector + Target	Effector	Effector + Target	Target	Effector	Effector + Target	Effector	Effector + Target	Targe
E:T Ratio	MQ	MQ+T	Lym	Lym+T	K_{562}	MQ	MQ+T	Lym	Lym+T	K_{562}	MQ	MQ+T	Lym	Lym+T	K_{562}
20:1	0.683 ± 0.284	0.628 ± 0.248	0.890 ± 0.201	0.761 ± 0.196	-----	0.721 ± 0.288	0.664 ± 0.281	0.741 ± 0.317	0.684 ± 0.271	-----	0.665 ± 0.344	0.598 ± 0.388	0.832 ± 0.208	0.809 ± 0.205	-----
10:1	0.596 ± 0.263	0.480 ± 0.256	0.787 ± 0.189	0.684 ± 0.195	-----	0.699 ± 0.284	0.507 ± 0.233	0.789 ± 0.286	0.523 ± 0.282	-----	0.579 ± 0.333	0.500 ± 0.320	0.699 ± 0.200	0.689 ± 0.172	-----
1:1	0.525 ± 0.243	0.401 ± 0.236	0.700 ± 0.185	0.588 ± 0.201	-----	0.699 ± 0.284	0.410 ± 0.266	0.789 ± 0.286	0.414 ± 0.259	-----	0489 ± 0.301	0.809 ± 0.256	0.615 ± 0.200	0.572 ± 0.181	-----
0:1	-----	-----	-----	-----	0.222	-----	-----	-----	-----	10.90	-----	-----	-----	-----	0.840

Table 2. Expression and release of sFasL by two groups of effecter cells, no significant difference was seen between two groups.

Groups	Zymosan		Betaglucan	
Test	Lymphocyte	Macrophages	Lymphocyte	Macrophages
Released Soluble Fas ligand	0.177±0.011	0.170±0.009	0.176±0.015	0.173±0.0131

DISCUSSION

The first investigation on polysaccharide immune-modulators was published about 60 years ago (17). It was started with Zymosan, which increased resistance to bacterial and viral infections and promoted allograft rejection of tumor. Later, several studies focused on the anticancer effects of Betaglucan. Many researchers have extensively investigated Betaglucan. It was shown that Betaglucan could enhance macrophage production and increase nonspecific host resistance against tumor growth. The actual glucan receptor on macrophages has been identified, which mediates a broad spectrum of immune-pharmacological activities (18,19).

Betaglucan interacts with surface receptors of macrophages and NK cells and triggers the activation processes, a cascade of interactions and reactions initiated by macrophage regulatory factors treated with Zymosan and Betaglucan, leading to the potentiation of cytotoxic events in the cell culture systems. Effects of these stimulators on phagocytes and adherent functions of monocytes are also well established.

Some reports have focused on the mechanisms by which Zymosan and Betaglucan induce cytotoxic activities against tumors, including the release of TNF-α and other

cytokines (10,11). Zymosan and Betaglucan also directly evoke cytotoxic responses, mediated by MDMs and lymphocytes, especially NK cells.

Although we found no significant difference between Zymosan and Betaglucan in induction of MDMs and lymphocytes, in a non-specific manner, Betaglucan helps to boost the immune mechanisms against target cells by triggering a complex cascade of events leading to a fully armed of the immune effector system. This may reflect the fact that Zymosan and Betaglucan have different effects on target killing system.

Our results revealed that Zymosan and Betaglucan had immune-stimulatory effects on cytotoxic reactions to produce sFasL. Introduction of Zymosan to effector-target system induced the cytotoxic responses significantly, compared with control. Betaglucan also had a similar effect on effector cells especially on MDMs.

Our study is the first report providing evidence on ability of macrophages to secret sFasL (20-28) Another unexpected finding was the negative effect of Betaglucan on K562 leukemic cell line when stimulated by Betaglucan alone and It is similar to the effect of Betaglucan on MDMs, which can be explained by the identical origin of both cell lines from the bone marrow myeloid progenitor. Existing reports support the direct anti-cancer effects of Betaglucan on solid tumors. In conclusion, Zymosan and Betaglucan as immune stimulators are capable of inducing the release of sFasL consequently increasing the killing capacity of the immune effector cell; in inflammation and chronic infection.

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