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Zymosan induces selective release of arachidonic acid from rabbit alveolar macrophages via stimulation of a β-glucan receptor

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Zymosan, which is composed primarily of α-mannan and β-glucan polymers, is a well recognized activator of macrophages. The type receptor by which unopsonized zymosan induces arachidonic acid release was investigated. It was found that particulate β-glucan and zymosan stimulated an identical dose-dependent release of arachidonic acid. This release of arachidonic acid by zymosan was blocked by soluble β-glucans whereas soluble mannan had no effect. This inhibition was not due to a general toxic effect of the soluble β-glucans as they had no effect on calcium ionophore-induced release of arachidonic acid. β-glucan-induced fatty acid release from these cells was shown to be fairly specific for arachidonic acid. These data reveal that zymosan stimulates the specific release of arachidonic acid from rabbit alveolar macrophages, at least in part, via a β-glucan receptor.

Zymosan; β-Glucan; α-Mannan; Alveolar macrophage; Arachidonic acid metabolism

1. INTRODUCTION

Zymosan, a particulate cell wall product of the yeast *Saccharomyces cerevisiae*, is a well-recognized activator of alveolar macrophages (AMØ). Although particulate zymosan is a potent stimulator of the alternative complement pathway [1] and is therefore readily opsonized, unopsonized zymosan is also a potent activator of AMØ. Unopsonized zymosan is phagocytosed and stimulates the secretion of lysosomal enzymes [2] and reactive oxygen intermediates [3] as well as the release of arachidonic acid (AA) from the membrane phospholipids and its subsequent conversion to a variety of metabolites [4,5]. Zymosan contains some protein and lipid but polysaccharides account for 73% of its dry weight. This carbohydrate consits entirely of β-glucan and α-mannan (74% and 26% of total polysaccharide, respectively) [6]. Because AMØ possess separate receptors for both the mannan and glucan components of zymosan [7], it is not clear which receptor is involved in the stimulation of AA release from these cells by zymosan. The purpose of this study was to determine which of these two receptors mediates zymosan-induced AA release in rabbit AMØ.

2. MATERIALS AND METHODS

2.1. *Materials*

^{14}C-fatty acids were obtained from New England Nuclear. Zymosan, particulate β-glucan and α-mannan from *S. cerevisiae*, β-glucan from barley and the algal β-glucan, laminarin, were all purchased from Sigma. All of the commercially obtained carbohydrate polymers were used without further purification. Zymosan was prepared in calcium and magnesium-free Hank's balanced salt solution (HBSS) according to the method of Bonney et al. [8]. Particulate β-glucan was prepared by suspension in HBSS at 5 mg/ml. Both zymosan and particulate β-glucan were subjected to tip sonication just prior to use to separate any adherent particles. Following sonication, particles were counted in a hemacytometer. β-glucan from barley is only partially soluble in water. The soluble portion was isolated following centrifugation at 15,000 × *g* as performed by Czop and Austen [9].

2.2. *Cell isolation*

AMØ were isolated from New Zealand white rabbits by bronchoalveolar lavage as previously described [10]. Cells isolated by this procedure were > 95% AMØ as determined by morphology. Following isolation, the AMØ were allowed to adhere to Costar 6-well or 24-well tissue culture plates at a concentration of 1.56×10^5 cells/cm^2. After 60 min in a fully humidified atmosphere of 95% air/5% CO_2, the wells were extensively washed to remove any non-adherent cells.

2.3. *Cell labeling*

The adherent AMØ were incubated with 0.1 μCi of ^{14}C-AA per well in a 1:1 mixture of Medium 199 and RPMI 1640 supplemented with 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin and 0.1% fatty acid-free bovine serum albumin (culture medium) for 2 h at 37°C in fully humidified 95% air/5% CO_2. Following incubation, the wells were washed three times with HBSS to remove any unincorporated [^{14}C]AA.

2.4. *Dose–response for AA release mediated by zymosan or particulate β-glucan*

[^{14}C]AA labeled AMØ in 6-well plates were covered with 0.9 ml culture medium and stimulated by addition of 0.1 ml of various concentrations of zymosan or particulate glucan in HBSS. After 90 min at 37°C, measurement of [^{14}C]AA release was performed as previously described [5]. Basal release was determined with AMØ cultures exposed to HBSS alone.

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2.5. *Dose-response for inhibition of zymosan-induced AA release by soluble α-mannan or β-glucan*

To investigate the inhibition of zymosan-induced AA metabolism by soluble β-glucan or α-mannan, [^{14}C]AA labeled AMØ in 24-well plates were overlayed with 0.45 ml of culture medium and preincubated with 0.05 ml of variable concentrations of either α-mannan, soluble barley β-glucan or laminarin for 30 min at 37°C. The concentrations of mannan, barley β-glucan or lamanarin used in these studies were expressed as μg/ml as determined by the anthrone method using a standard curve based on glucose [11]. The release of [^{14}C]AA was then initiated by addition of 2×10^7 particles of zymosan in 0.05 ml HBSS and quantified after 90 min of stimulation.

2.6. *Effect of soluble glucans on A23187 induced AA release*

[^{14}C]AA labelled AMØ in 6-well plates were overlayed with 0.8 ml of culture medium and preincubated with 0.1 ml of lamanarin or barley β-glucan for 30 min at 37°C. The release of [^{14}C]AA was then initiated by the addition of the calcium ionophore A23187 in 0.1 ml of HBSS. The final concentrations of lamanarin and barley β-glucan were 426 μg/ml and 319 μg/ml, respectively. The final concentration of calcium ionophore was 10 μg/ml. The release of [^{14}C]AA was quantified after 90 min of stimulation.

2.7. *Effect of the time of preincubation with soluble barley β-glucan on zymosan-mediated AA release*

To define the time course of the inhibition of zymosan-stimulated AA metabolism by soluble β-glucan, [^{14}C]AA labeled AMØ in 6-well plates were overlayed with 0.8 ml of culture medium and preincubated with 319 μg of barley β-glucan in 0.1 ml for various periods of time up to 40 min at 37°C. The cells were then stimulated with 2×10^7 particles of zymosan in 0.1 ml HBSS and the release of [^{14}C]AA quantitated after 90 min of stimulation.

2.8. *Determination of fatty acid release specificity*

AMØ in 24-well plates were labeled with [^{14}C]AA, [^{14}C]linoleic acid, [^{14}C]oleic acid, [^{14}C]stearic or [^{14}C]palmitic acid by the same procedure described above for labeling the cells with [^{14}C]AA with one exception. Because the uptake of linoleic, oleic, stearic and palmitic acid was approximately 4 times lower than the uptake of AA, the AMØ were labeled with 0.4 μCi of these four fatty acids rather than the 0.1 μCi used with AA. In a preliminary study to assess the cellular distribution of the radiolabeled fatty acids taken up by the AMØ, the cells were extracted and the complex lipids (phospholipids, sphingolipids and cardiolipin), neutral lipids, free fatty acids and cholestrol esters separated by thin layer chromatography. The percentage of fatty acid found in the complex lipid fraction by this analysis varied from a low of 52% for palmitic acid to a high of 87% for arachidonic acid. To measure the β-glucan-dependent release of fatty acid, the radiolabeled cells were overlayed with 0.8 ml culture medium and stimulated by the addition of 0.1 ml of particulate β-glucan at 2×10^8 particles/ml at 37°C. Release of [^{14}C]fatty acid was quantified after 90 min as previously described [5].

3. RESULTS AND DISCUSSION

Exposure of [^{14}C]AA labeled AMØ to either zymosan or particulate β-glucan resulted in the dose-dependent release of [^{14}C]AA from the membrane phospholipids. As can be seen in Fig. 1, both agonists evoked nearly identical maximal releases of [^{14}C]AA, 13.7 ± 0.9% (mean ± S.E.M.) for zymosan and 13.3 ± 0.5% for particulate β-glucan. Furthermore, the dose-response curves for these two agonists were virtually identical when compared on a particle-to-particle basis. EC_{50}'s were 5.3×10^6 particles/ml for zymosan and 5.6×10^6 particles/ml for particulate β-glucan. This translates to a particle-to-cell ratio of 3.6:1 required to achieve half-maximal stimulation with either particle.

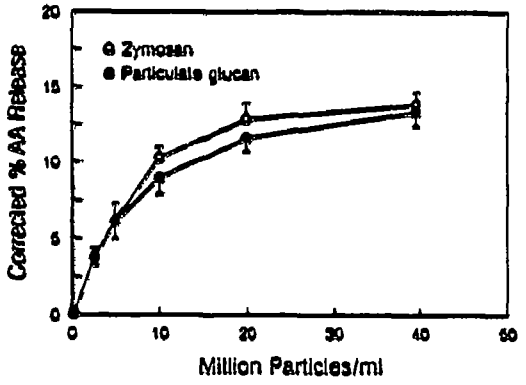

Fig. 1. Dose-response curves for zymosan particulate β-glucan stimulation of AA release. Shown in this figure are the dose-response curves for the release of [^{14}C]AA from labeled AMØ stimulated by either zymosan (open circles) or particulate β-glucan (closed circles) at the concentrations indicated for 90 min at 37°C. The values shown are the mean ± S.E.M. [^{14}C]AA released and are expressed as a percentage of the total [^{14}C]AA incorporated in the cells. All values have been corrected by subtracting the basal release of [^{14}C]AA. The data were obtained from three to five independent AMØ preparations.

These results suggested that zymosan stimulates AA release via interaction with a β-glucan receptor. To test this possibility, the ability of soluble β-glucan (either lamanarin or barley β-glucan) or α-mannan to inhibit zymosan-dependent AA release was investigated. As shown in Fig. 2, the soluble β-glucans had a significant dose-dependent effect whereas α-mannan had no effect

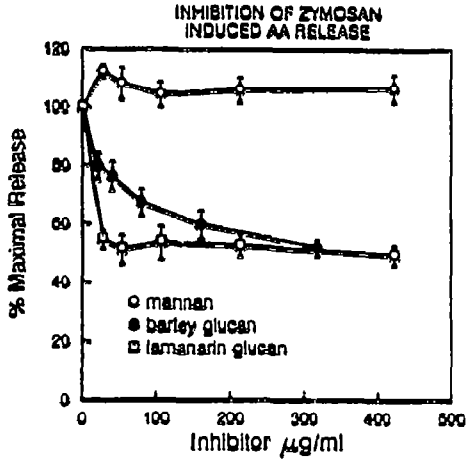

Fig. 2. Dose-response curves for the inhibition of zymosan-induced AA release by soluble mannan or β-glucans. Shown in this figure are the dose-response curves for the inhibition of zymosan-mediated [^{14}C]AA release by α-mannan (open circles), barley β-glucan (closed circles), or laminarin (squares). [^{14}C]AA labeled AMØ were preincubated with the indicated concentrations of yeast α-mannan, soluble barley β-glucan or the soluble algal β-glucan, laminarin, for 30 min at 37°C and then challenged with 2×10^7 particles/ml of zymosan for 90 min at 37°C. The values shown are the mean ± S.E.M. [^{14}C]AA released after 90 min of stimulation and are expressed as a percentage of the maximal [^{14}C]AA release observed in the absence of any inhibitor. The data were obtained from three to five independent AMØ preparations.

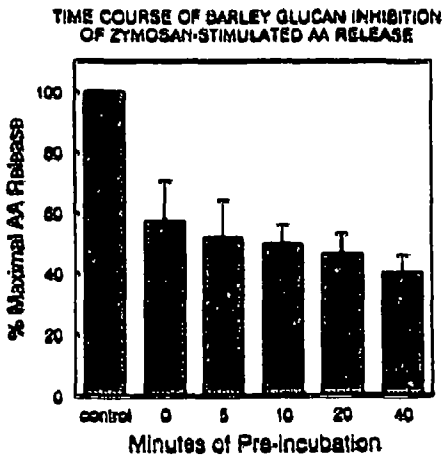

Fig. 3. Effect of the length of time of preincubation with barley β-glucan on the inhibition of zymosan-induced AA release. Shown in this figure is the effect of the length of preincubation of [^{14}C]AA labeled AMØ with barley β-glucan on the subsequent release of [^{14}C]AA upon stimulation with zymosan. [^{14}C]AA labeled AMØ were preincubated at 37°C with 319 μg/ml of soluble barley β-glucan for the periods indicated and then challenged with 2×10^7 particles/ml of zymosan for 90 min at 37°C. The values shown are the mean ± S.E.M. [^{14}C]AA released after 90 min of stimulation expressed as a percentage of the maximal [^{14}C]AA release observed in the absence of inhibitor. The data were obtained from three to five independent AMØ preparations.

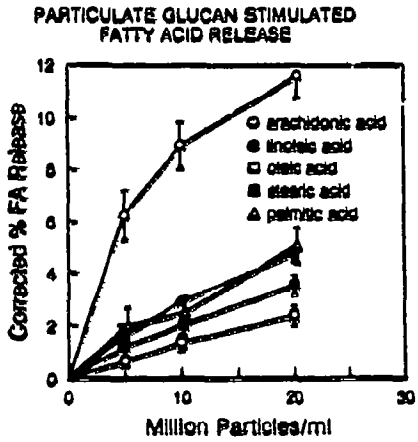

Fig. 4. Dose-response curves for particulate β-glucan stimulation of fatty acid release. Shown in this figure are the particulate β-glucan dose response curves for the release of radiolabeled fatty acids from AMØ. AMØ were prelabeled separately with [^{14}C]AA, [^{14}C]linoleic acid, [^{14}C]oleic acid, [^{14}C]stearic acid, or [^{14}C]palmitic acid and then challenged with the indicated concentrations of particulate β-glucan for 90 min at 37°C. The values shown are the mean ± S.E.M. [^{14}C]fatty acid released after 90 min of stimulation expressed as a percentage of the total [^{14}C]fatty acid incorporated in the cells. Total dpm incorporated for each fatty acid were: AA, 88,771 dpm; linoleic acid, 116,723 dpm; oleic acid, 71,796 dpm; stearic acid, 53,746 dpm; palmitic acid, 66,935 dpm. All values have been corrected by subtracting the basal release of the appropriate [^{14}C]fatty acid. The data were obtained from three independent AMØ preparations.

at all. Neither α-mannan nor the soluble β-glucans stimulated AA release above basal levels (data not shown). Maximal inhibition for both lamanarin and barley β-glucan was identical at about 50% of maximal release. Although maximal inhibition was identical, lamanarin, which shares more homology with the β-glucan contained in zymosan, was a more efficient inhibitor with maximal inhibition caused by 26.6 μg/ml as opposed to 319 μg/ml for barley β-glucan. The finding that only soluble β-glucans inhibited zymosan-dependent AA release from AMØ confirmed that zymosan was acting, at least in part, via a β-glucan receptor. It should be noted that maximal inhibition of zymosan-induced AA release by soluble β-glucans was only 50%. This suggests that another receptor exists which contributes to the release of AA following stimulation of the AMØ by zymosan. It is, however, clear that this other receptor is not the mannose receptor since α-mannan did not inhibit zymosan-induced AA release.

Table 1

Effect of soluble glucans on A23187-induced AA release

Inhibitor	% Release
Laminarin	104.25 ± 7.35
Barley β-glucan	100.70 ± 9.00

Each data point represents the mean ± S.E.M. of three independent cell preparations. Release of AA in the absence of soluble β-glucans was 15.53 ± 3.92% of total label.

To ensure that the inhibitory action of the soluble β-glucans on zymosan-induced AA release was not due to a general toxic effect on the cell, the effect of the soluble β-glucans on A23187-induced AA release was examined. As can be seen from Table I, neither lamanarin or barley β-glucan had any effect on the release of AA stimulated by A23187. This indicates that the inhibition of zymosan-induced AA release by the soluble β-glucans was not due to inhibition of the phospholipases involved in AA release.

To determine whether inhibition of AA release by soluble β-glucan required endocytosis of the receptor, AMØ were preincubated with soluble β-glucan for various periods of time and then challenged with zymosan. Although there was a trend toward slightly increased [^{14}C]AA release with shorter times of preincubation (Fig. 3), significant inhibition was observed even when the soluble glucan and zymosan were added simultaneously (time zero). When the soluble β-glucan and zymosan were added simultaneously, the release of [^{14}C]AA was still only 57.2 ± 13.5% of maximal release obtained in the absence of soluble β-glucan. This suggests that the mechanism of inhibition of zymosan-induced AA release from rabbit AMØ by soluble β-glucan does not require receptor endocytosis. This result is in contrast to those reported by Goldman [12] with mouse peritoneal macrophages and Czop [13] with human monocytes. These investigators found that, in terms of the phagocytosis of zymosan, the rate of inhibition by solu-

ble β-glucans was most consistent with endocytosis of the receptor.

The release of AA observed following stimulation of the β-glucan receptor could reflect a process specific for AA, or possibly, a more generalized release of fatty acids from the macrophage membrane phospholipids. To address this question, alveolar macrophages were independently loaded with radiolabelled palmitic, stearic, oleic, linoleic or AA, and stimulated with β-glucan. Although these five fatty acids account for more than 95% of the fatty acids esterified in the phospholipids of rabbit alveolar macrophages and are present in approximately equal quantities [14], stimulation of the β-glucan receptor with 2×10^7 particles/ml of particulate β-glucan resulted in the release of AA that was significantly greater than that observed for the other four fatty acids. As depicted in Fig. 4, 11.6 ± 0.8% of total labeled AA was released compared to less than 5% for linoleic, oleic, stearic and palmitic acid. These results indicate that stimulation of the β-glucan receptor leads to the selective release of AA from the macrophage membrane phospholipids. Since β-glucans are components of the cell walls of many pathogenic fungi, stimulation of resident alveolar macrophage β-glucan receptors with resulting release and metabolism of AA may play an important role in the pathogenesis of pulmonary fungal infections.

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