AN INVESTIGATION INTO THE EFFECT OF LIPOPOLYSACCHARIDE AND (1→3)-β-D-GLUCAN EITHER IN COMBINATION OR SEPARATELY ON THE DEGRANULATION OF MAST CELLS ISOLATED FROM THE GUINEA PIG LUNG AND THE RAT PERITONEAL CAVITY

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Abstract

Mast cells are of central importance to the immediate and chronic effects of lung inflammation typified by bronchial hyperreactivity, through release of inflammatory mediators including histamine. This work examines the histamine releasing effect of Lipopolysaccharide and (1→3)-β-D-glucan, both of which are found in cotton and other organic dusts, on mast cells isolated from the rat peritoneum and guinea pig lung.

Introduction

Exposure to the indoor environment of buildings contaminated by organic dust, mould and damp conditions has been shown to lead to a range of adverse health conditions, in particular respiratory disorders. The measure of this contamination is by an often overlooked index, namely indoor air quality. The increasing use of air conditioning and changes in the ventilation systems often results in the degradation of the air we breathe, not evident in the past when draughtier buildings were more commonplace. Air contamination by microbially derived agents is implicated in a range of diseases including extrinsic allergic alveolitis, humidifier fever, asthma, rhinitis, sick building syndrome, hypersensitivity pneumonitis and organic toxic dust syndrome (Leslie and Lunau 1991, Rylander and Jacobs 1994).

A number of key studies have identified Endotoxin (also referred to as Lipopolysaccharide LPS) derived from gram negative bacteria as being of importance to the pathology of a range of organic dust related diseases. More recently β(1,3)-D-glucans derived from the inner cell wall of various yeasts and plant and belonging to a class of compounds known as biological response modifiers (BRM) having tumouricidal properties material have received a great deal of interest. However these glucose polymers are also found in areas of organic dust and microbiological contamination and there is increasing evidence that they may be involved in various inflammatory conditions of the respiratory system (Fogelmark et. al. 1994, Milanowski 1997).

The mast cell is known to play an important role in airway hypperresponsiveness particularly in bronchial asthma and hay fever, and recent evidence suggests that they are also important in the chronic inflammatory processes. Mast cells may be activated immunologically via cross-linking of surface bound IgE and by non-immunological means through various methods of direct agent activation. Bacterial, fungal and viral products have been shown to vary the release of mediators from the mast cell (Norn et. al. 1987). These cells produce a wide range of mediators including biogenic amines, heparin, proteases, prostaglandins, leukotrienes, kinins and cytokines (Metcalfe et.al. 1997). In addition they are found in almost every organ system in the human body being in close proximity to blood and lymphatic vessels, nerves and beneath epithelial surface such as those of the lung. This is an ideal location for interaction with the external environment carrying pathogens and environmental pollutants such as bacteria, fungi and their breakdown products. Owing to its location and the varied range of mediators produced, the mast cell is not only ideally situated for producing the acute phases of respiratory distress but may also be of vital importance to orchestrating the more chronic conditions of various disorders of the pulmonary system.

Therefore the mast cell has been chosen as a key cell to study to more fully understand the cellular mechanisms involved in organic dust related respiratory diseases. The pathophysiology of such diseases is poorly understood and investigation of the interaction of dust and mould contaminants with mast cells should provide valuable information as to the mechanisms of such diseases.

The work presented here briefly outlines some of the studies carried out in our laboratory to examine the effect of two of these agents LPS and Bakers Yeast glucan on the degranulation of mast cells obtained from the rat peritoneum, a connective tissue type mast cell, and guinea pig lung, a mucosal type mast cell (Church et. al. 1997), by analysing the release of the preformed mediator histamine.

Materials and Methods

Animals

Male Dunkin-Hartley guinea pigs weighing 250-500g and male Sprague-Dawley and Wistar rats 200-400g were obtained through the joint animal services department of Cardiff University.

Materials

All materials unless otherwise stated were purchased from the Sigma Chemical Company and diluted in HEPES-tyrode buffer. Sheep anti-rat IgE came from ICN Biochemicals.

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Methods

Mast Cell Isolation (Ali and Pearce 1985).

Guinea pig lung mast cells (GPLMC). Guinea-pigs were euthanized with sodium pentobarbitol. The lungs were removed and chopped into 1-2mm pieces and placed in buffer containing mM N-2-Hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES) 10, $CaCl_2.5H_2O$ 1, NaH_2PO_4 0.4, NaCl 137, glucose 5.6 Collagenase was added at 20IU/ml with BSA at 1mg/ml and incubated with mixing at 37°C for 90 minutes. After which the mixture was filtered through Nylon gauze (Fisher) pore size 150µm. The cell mixture was centrifuged at 200g for 5-6 minutes at 4°C. The cells were then centrifuged similarly and resuspended in warm buffer for use in experiments.

Rat peritoneal mast cells (RPMC). Where rat sensitisation was used it was carried out by intraperitoneal injection with 100µg/ml ovalbumen and aluminum hydroxide 100mg/ml (as adjuvant) was used.

The rats were killed by CO_2 asphyxiation. The peritoneum was lavaged with 20-40ml of HEPES-Tyrode buffer containing heparin (5IU/ml) and BSA (1mg/ml). The lavage fluid was centrifuged at 200g for 5 minutes twice, and the cell pellet resuspended in HEPES buffer for use in experiments.

In the case of purified mast cells two systems were employed either by percoll density gradient centrifugation and the other using metrizamide. In the case of percoll a 30/80% density gradient was employed (Leal-Berumen et. al. 1994) with cells being suspended in calcium free RPMI and spun for 15 minutes at 800g and 4°C. Metrizamide centrifugation (Dileepan et. al. 1989) was carried out by layering the cell solution on 4ml of 22.5% metrizamide solution in calcium free HEPES buffer. After purification the cell pellet containing the mast cells was resuspended and spun twice for a further 5 minutes at 200g. Mast cell purity was assessed by alcian blue staining (Gilbert and Ornstein 1965) or cytospin stained with Leishmanns stain - typically purity was in excess of 90% as was cell viability assessed by trypan blue staining.

Incubation with Various Agents

These experiments were carried out using 150µl of the various cell isolates to which 30µl of the agent to be examined and 150µl buffer was added. The tubes were incubated at 37°C for either 15 or 30 minutes. The reaction was stopped by addition of excess cool buffer thereby diluting the mixture. The mixture was immediately centrifuged at 200g for 2minutes and the supernatant poured off. The cell pellet was resuspended in an equivalent amount of buffer. Finally the supernatant and cell pellet proteins were precipitated using concentrated perchloric acid and the resulting mixtures frozen until assaying for histamine content.

Histamine Fluorescent Assay (Shore et. al. 1959)

To remove contaminating proteins such as spermidine from the tubes containing used to analyse histamine content of the guinea-pig lung mast cells, a micro-manual procedure was developed substituting heptanol (Redlich and Glick 1965) for the usual solvent system of butanol/heptane for the separation of histamine from pellet/supernatant mixtures. Various other steps in the usual procedure were changed to improve the precision which was of the order of 10-15%.

After a number of extraction steps, 0.1% o-pthalaldehyde (OPT) was added to the extracted histamine under basic conditions at 4°C for 30 minutes, after which the reaction was stopped by addition of phosphoric acid, this produced a stable fluorophore via a schiffs base mechanism. The reaction was carried out in ice as this increased the stability of the resultant fluorophore. Fluorescence was measured using a Perkin-Elmer LS-5 fluorimeter set at wavelengths for excitation 360nm and emission 440nm.

Each experiment also included a set of calibrators and standards to ensure the validity of each assay as well as the reproducibility of the results.

A similar procedure was adopted for the analysis of histamine content in the rat peritoneal lavage tubes without the requirement for extraction owing to the lower levels of contaminants. Similarly a micro-manual procedure was used with a fluorophore being produced by reaction of histamine with 0.1% OPT under basic conditions for 4 minutes at room temperature with the reaction being stopped by addition of phosphoric acid.

Electron Microscopy

Negative staining electron microscopy was carried out with the assistance of Dr A.C. Hann at the microscopy unit of Cardiff University. Briefly the technique used to visualise the cell shown was by transmission electron microscopy applying negative staining with uranyl acetate.

Results

Histamine release was determined by calculating the percentage histamine in the supernatant based on the total in the cell pellet and supernatant histamine after correction for spontaneous release. Spontaneous release for the results shown was typically below 10%.

Results shown as means ± SEM for the number of observations stipulated (* denotes significance at $p > 0.05$ unless otherwise stated calculated by the students t-test).

GPLMCs showed a definite but small increase on the addition of LPS at 100µg/ml whereas Bakers Yeast glucan (BY glucan) was noticeably less effective Tables 1 and 2.

In both RPMC and GPLMCs it may be seen that when applied in combination, BY glucan/LPS caused an increase of up to three times the histamine release when compared to the compounds alone (Table 3 and Figure 2). However it should be noted that this is apparently not the case in the purified RPMC system suggesting that other cells assist in activating the mast cells further during the incubation.

In RPMCs, glucan exhibited a dose response relationship (Figure 1). Of the other polyglucose molecules tested only, dextran showed any effect - being 6-fold less than BY glucan. Also there was noticeable interaction between the cell membrane of the RPMC and BY glucan ,as visualised by electron microscopy. This most likely results in the activation of the mast cell and is probably antibody independent as the sensitised mast cells do not result in an increase in the level of activation on incubation with sheep anti-rat IgE (see Figure 5). The histamine release data presented demonstrates that this effect is likely to be potentiated by the addition of LPS. However, in all cases the data must be treated with some caution as the effects of the other cells in the incubation system may well contribute to the observed results (Figure 2), where purified RPMCs show very low histamine release compared to the similar effect of BY glucan/LPS combinations on unpurified rat peritoneal cell mixtures.

There appears to be a significant relationship between the absence of calcium ions and the histamine releasing effects of BY glucan (Figure 4). Unusually the presence of calcium appears to be inhibiting the releasing effects of BY glucan especially at the lower concentrations.

Discussion

The GPLMC and RPMC results suggest that the two agents, BY glucan and LPS in combination may synergistically cause an increase in histamine release and in turn mast cell activation. The presence of other cells in the incubation mixture may also activate the mast cells and account for some of the variability in the results. In the case of the RPMCs removal of the contaminating cells reduced the ability of most of the BY glucan/LPS combinations to release histamine, suggesting that other cells in the incubation mixture were important to the release of histamine observed.

It is important to note that BY glucan can significantly activate rat peritoneal mast cells and cause the release of histamine up to 75% of the total available for release, preliminary data examining such glucan levels on guinea-pig lung mast cells also releases high levels of histamine (data not shown). This is much greater than that achieved by known polyglucose activators such as dextran or other glucans including curdlan. However it is found that this effect is highly variable and may be due to the interaction of other cells in the system or the result of the particular glucan used, and indeed there has been differences between batches observed (data not shown). LPS was found to release virtually no histamine on its own in agreement with work carried out by other groups (Leal-Berumen et. al. 1994).

In the case of RPMCs of particular interest is the observation that the removal of calcium ions from the extracellular medium rather than blocking the release of histamine by BY glucan potentiated the effect even at low glucan concentrations. It is feasible that the destabilisation of the mast cell in calcium free buffer increases the ability of glucan to release histamine. Further study using various calcium channel blockers and known antagonists of histamine release from mast cells is underway to more fully understand this effect. Notably it has been observed by others working in this field that a glycoprotein, known as mast cell function associated antigen (MAFA), identified on a rat mucosal type mast cell line RBL-2H3and similar in structure to the calcium dependent animal lectins (C-type lectins), is able to bind various carbohydrates in particular mannose residues and in turn inhibit the antigenic release of histamine. It is feasible that BY glucan binds a similar receptor and this results in the inhibition of the histamine release observed in the presence of calcium ions (Binsack and Pecht 1997).

The observation of direct interaction between BY glucan and the rat peritoneal mast cell as visualised by the electron microscope is an important one, suggesting that the binding of glucan occurs at the surface, thus resulting in the activation of the mast cell by either receptor mediated or non-specific means.

It is possible that the mechanism involved in the release of histamine from the rat peritoneal mast cells by BY glucan is not via crosslinking of the FcεRI receptor, as there is a decrease in histamine release in the presence of calcium and anti-IgE. It is likely that some other receptor is involved, as well as non-specific interactions at the cell membrane. A similar mechanism has been suggested for dextran (Metcalfe et. al. 1997). Furthermore other cells in the peritoneal lavage or tissue derived cell isolate may influence the activation of the mast cells, in particular macrophages which are known to bind glucan at the complement type 3 (CR3) receptor (Milanowski 1997).

In conclusion the results provide important additional data establishing a possible link between BY glucan/LPS and mast cell activation, showing the effect to be multifactorial, variable and complex. As a result of the diverse range of mediators that can be released by the mast cell this will in turn influence acute and chronic inflammation, and in particular the respiratory and mucosal disorders caused by exposure to the air in dusty, damp and mouldy buildings. Further work examining the effect of BY glucan and LPS on the release of other mediators, particularly cytokines, is necessary to more fully understand the role of the mast cell in organic dust related respiratory disorders.

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Table 1. Incubation for 30 minutes at 37°C of cell isolate with Bakers Yeast glucan (n=5)

Stimulus	Histamine Release %
glucan 100µg/ml	3.9 ± 1.8
glucan 10µg/ml	3.7 ± 0.6
glucan 1µg/ml	2.9 ± 0.9
glucan 0.1µg/ml	0.1 ± 0.4

Table 2. Incubation for 30 minutes at 37°C of cell isolate with LPS (n=5)

Stimulus	Histamine Release %
LPS 100µg/ml	8.7 ± 5.1
LPS 10µg/ml	4.3 ± 4.1
LPS 1µg/ml	3.8 ± 2.9
LPS 0.1µg/ml	3.6 ± 4.0

Table 3. Incubation for 30 minutes at 37°C of cell isolate with LPS/ Bakers Yeast glucan combinations (n=5)

Stimulus	Histamine Release %
glucan 100µg/ml LPS 100µg/ml	12.2 ± 8.0
glucan 100µg/ml LPS 10µg/ml	13.6 ± 8.6
glucan 100µg/ml LPS 1µg/ml	1.3 ± 1.0
glucan 10µg/ml LPS 100µg/ml	13.4 ± 6.6
glucan 10µg/ml LPS 10µg/ml	6.8 ± 3.4
glucan 10µg/ml LPS 1µg/ml	17.2 ± 6.0
glucan 1µg/ml LPS 100µg/ml	12.2 ± 3.5
glucan 1µg/ml LPS 10µg/ml	11.2 ± 5.4
glucan 1µg/ml LPS 1µg/ml	12.7 ± 4.9

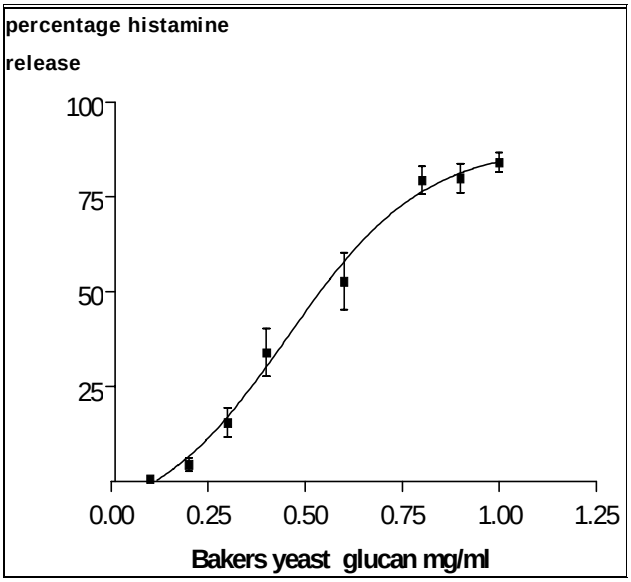

Figure 1: Unsensitised rat peritoneal mast cell histamine release at varying Bakers Yeast glucan levels (n=5)

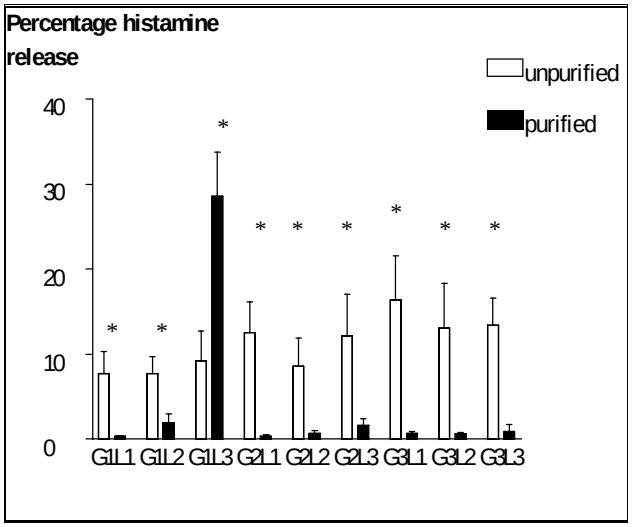

Figure 2: Comparison of histamine release of varying Bakers yeast glucan and LPS concentrations (n=3-5)
(G1=100µg/ml, G2=10µg/ml, G3=1µg/ml, L1=100µg/ml, L2=10µg/ml, L3=1µg/ml)

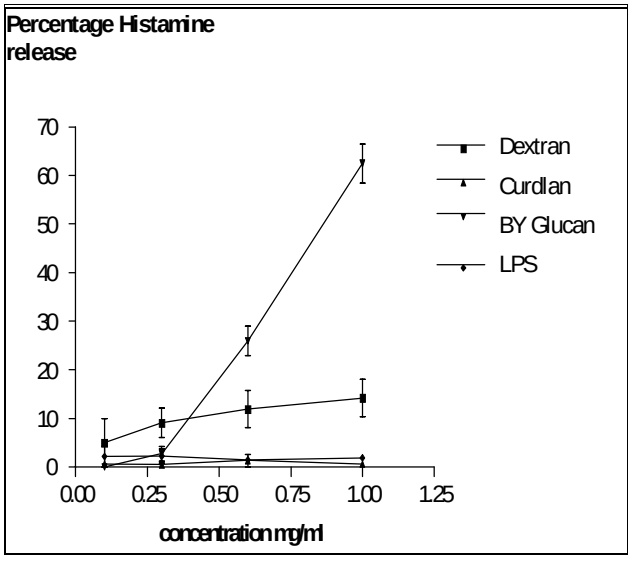

Figure 3: Comparison of histamine release of different agents with non sensitised RPMCs (n=3-5)

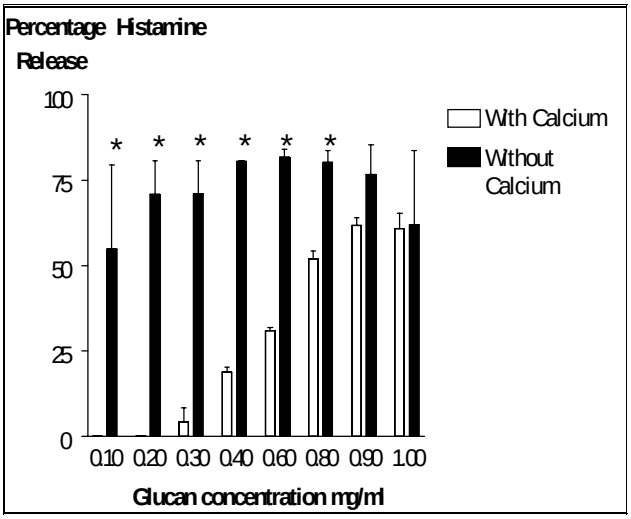

Figure 4: Comparison of the histamine release elicited by BY glucan with or without 0.1mM Calcium ions on unsensitised RPMCs (n=3-5)

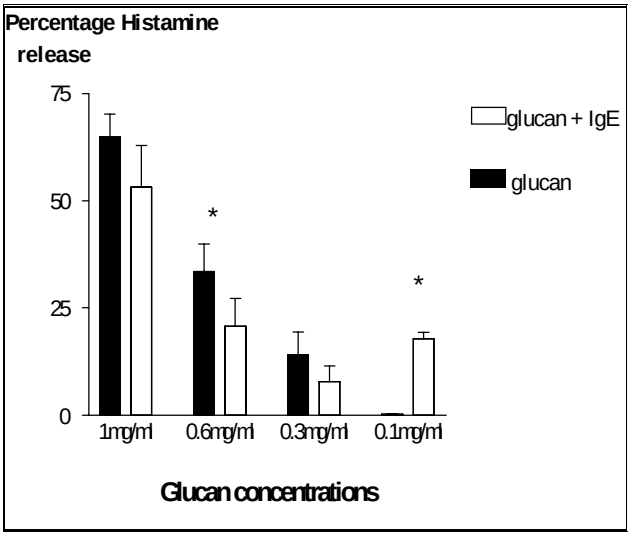

Figure 5: Histamine release on incubation of BY glucan with or without 1:500 anti-IgE dilution for sensitised RPMCs (n=3-5, also a 1 in 500 dilution causing a histamine release of 41.7 ± 7.8%)

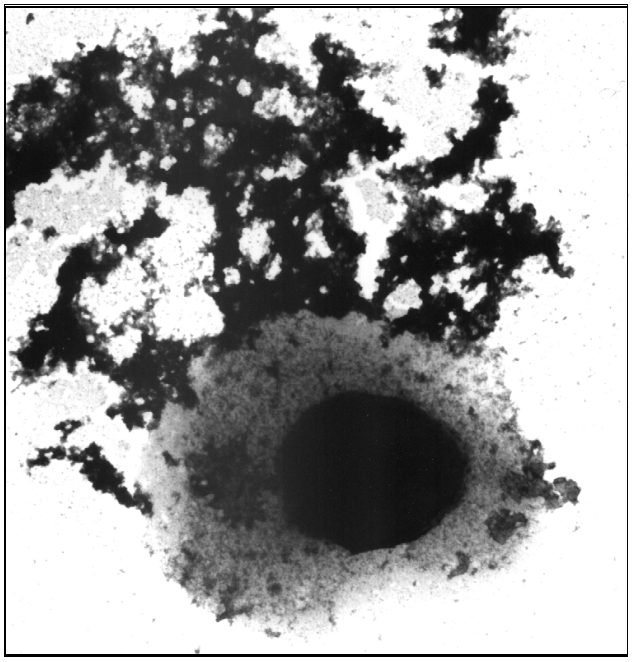

Figure 6: Electron Micrograph of the a negatively stained mast cell interacting with glucan from Bakers Yeast (magnification x10 000).