

## BACTERIAL ENDOTOXIN ASSOCIATED WITH POLLEN AS A POTENTIAL FACTOR AGGRAVATING POLLINOSIS

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**Abstract:** Eight samples of allergenic pollen originating from five species of anemophilous plants were investigated in order to estimate their contamination with Gram-negative bacteria and bacterial endotoxin. The concentration of Gram-negative bacteria ranged from 0 to 33000 cfu/g and the concentration of endotoxin ranged from 3.75 to 37.5 ng/mg. The concentration of endotoxin did not correlate to that of viable Gram-negative bacteria. It is suggested that endotoxin may play a role in pathophysiology of pollen allergy.

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### INTRODUCTION

Gram-negative bacteria developing on plant and animal surfaces or decomposing organic matter, produce endotoxin which is a known airborne immunotoxicant causing inflammatory reactions in the lungs of exposed men and animals [3, 18]. Depending on the inhaled dose, endotoxin is believed to evoke toxic pneumonitis, chronic bronchitis, mucous membrane irritation, or to aggravate the adverse pulmonary reactions caused by exogenous allergens [15, 16]. Michel *et al.* [17] have demonstrated that small amounts of endotoxin occurring in house dust may aggravate the symptoms of house dust asthma. As, to the best of our knowledge, this problem has not so far been investigated in relation to pollinosis, we have begun a study of the possible role of bacterial endotoxin in pathomechanism of this disease. The aim of the initial study presented here was to determine whether pollen grains could be contaminated with endotoxin in amounts sufficient to influence clinical course of pollinosis.

### MATERIALS AND METHODS

Eight pollen samples of five plant species were examined during May/June 1995 to determine concentrations of endotoxin and Gram-negative bacteria. The samples were collected during the flowering seasons of 1994 and 1995 from the following plants: 1) rye (*Secale cereale*) in 1994; 2) mugwort (*Artemisia vulgaris*) in 1994; 3) hazel (*Corylus avellana*) in 1995; 4) European alder (*Alnus glutinosa*) in 1995 - all from ecologically clean regions in Kraków Province (Southern Poland). Additionally, white warty birch (*Betula verrucosa*) pollen was collected four times - 5) in 1994 (lot # 1) and again 6) in 1995 (lot # 2) from an ecologically clean region in Kraków Province, 7) in 1995 (lot #3) from a housing estate located in the City of Kraków, and 8) in 1995 (lot #4) from a polluted industrial area near a cokery in Kraków. The following technique of pollen sampling was used: the flower-bearing stems (branches) were cut before starting pollination and then placed in flagons into sterile

**Table 1.** Concentration of bacterial endotoxin and Gram-negative bacteria in samples of pollen grains from various plants

Pollen sample	Harvesting year	Place of collecting pollen	Concentration of endotoxin (ng/mg)	Concentration of Gram-negative bacteria (cfu/g)
Rye ( <i>Secale cereale</i> )	1994	ecologically clean region	3.75	5500
Mugwort ( <i>Artemisia vulgaris</i> )	1994	ecologically clean region	37.50	2500
Hazel ( <i>Corylus avellana</i> )	1995	ecologically clean region	7.50	0
Alder ( <i>Alnus glutinosa</i> )	1995	ecologically clean region	7.50	11500
Birch ( <i>Betula verrucosa</i> ) - lot # 1	1994	ecologically clean region	7.50	15000
Birch ( <i>Betula verrucosa</i> ) - lot # 2	1995	ecologically clean region	7.50	500
Birch ( <i>Betula verrucosa</i> ) - lot # 3	1995	housing estate	7.50	33000
Birch ( <i>Betula verrucosa</i> ) - lot # 4	1995	neighbourhood of a cokery	7.50	0

chambers and allowed to pollinate. The falling pollen was collected onto a sterilised foil and was then kept deep-frozen until the examination. Concentration of endotoxin in the samples of pollen grains was determined using the Limulus Amebocyte Lysate (LAL) gel tube test, as described previously [10]. Briefly, the pollen samples were suspended in sterile and pyrogen-free 0.9% NaCl solution in proportion 100 mg /10 ml, heated to 100°C in a Koch apparatus for 15 minutes, and after cooling serial dilutions were made. The 0.1 ml dilutions were mixed equally with the LAL reagent (“Pyroquant”, Pyroquant Diagnostik GmbH, Germany). The test was incubated for one hour in a water bath at 37°C using pyrogen-free water as a negative control and standard *Escherichia coli* EC-5 endotoxin (“Pyrotell”, Pyroquant Diagnostik GmbH, Germany) as a positive control. Each sample was examined in duplicate. Results were reported as nanograms of the equivalents of the *E. coli* endotoxin per milligram of pollen. To convert to Endotoxin Units (EU), multiply the value in nanograms by 5.

The concentration of Gram-negative bacteria in the pollen samples was determined by dilution plating. One hundred milligrams of each sample was suspended in 10 ml sterile 0.9% NaCl solution containing 0.1% (v/v) of Tween 80. After vigorous shaking, 10-fold serial dilutions were made up to 10<sup>-4</sup>. The 0.1 ml aliquots of each dilution were spread on duplicate sets of eosin methylene blue agar plates (EMB, Difco). The plates were incubated first for one day at 37°C, then for three days at 22°C and for the next three days at 4°C [10]. Colonies were counted and differentiated using API Systems for identification of fermenting (API 20E) and non-fermenting (API NE) Gram-negative bacteria (API Analytab, Plainview, NJ, USA). The data were reported as colony forming units (cfu) per gram of pollen. Statistical analysis of the possible correlation between Gram-negative bacteria and endotoxin concentrations was carried out using the Statgraphics 5.0 package (Spearman rank correlation coefficient).

## RESULTS

The concentration of endotoxin in the examined samples of pollen was within the range 3.75–37.5 ng/mg, being the lowest in rye pollen and the highest in mugwort pollen (Tab. 1). The most common value was 7.5 ng/mg which was found in pollen samples of hazel and alder, as well as in all four samples of birch pollen. The concentration of Gram-negative bacteria was within the range 0–33000 cfu/g. No Gram-negative bacteria were cultured from the hazel pollen and birch pollen sampled at the coking plant (lot #4), whereas the highest concentration was observed in the sample of birch pollen from housing estate (lot #3). The only species of Gram-negative bacteria was *Pantoea agglomerans* (synonyms: *Erwinia herbicola*, *Enterobacter agglomerans*), with the exception of one colony of *Acinetobacter* sp., cultured from the mugwort pollen sample. There was no significant relationship between the concentration of endotoxin and the concentration of Gram-negative bacteria ( $p = 0.77$ ).

## DISCUSSION

To date little attention has been paid to the possible contribution of the microflora associated with the pollen grains to the overall allergenic effect of pollen. Colldahl and Carlsson [6] demonstrated in 1968 that patients allergic to pollens reacted to specific pollen extracts, as well as to extracts prepared from microorganisms (a fungus *Cryptococcus luteolus* and a Gram-negative bacterium *Pseudomonas maltophilia*) cultured from samples of these pollens. In the case of *Cryptococcus luteolus*, the authors supported their clinical observation by results of the Ouchterlony test. Using scanning electron microscopy, Colldahl and Nilsson observed bacteria and fungi on surfaces of pollen grains [7]. To the best of our knowledge, no further studies on the possible role of Gram-negative bacteria in pollen allergy were undertaken, and the problem of the potential effects of bacterial endotoxin associated with pollen has never been studied.

The levels of endotoxin found by us in the samples of pollen grains were comparable with the values reported for wood [10], dust from silage [20], dusts from pig farms [8] and hen farms [5, 24]. Chronic exposure to these materials could be a cause of work-related pulmonary diseases, with bacterial endotoxin being one of the suspected etiological agents [15, 22]. Our values were 10–1000 times lower compared to dusts from grain, herbs, hay and the sapwood of pine that show a very strong endotoxic potency [9, 10, 11, 12, 20].

To estimate the exposure to pollen-bound endotoxin we have chosen the typical case of birch pollen. It could be calculated that the endotoxin amount on each birch pollen grain weighing 9.48 ng [13] is  $7.11 \times 10^{-5}$  ng. As the peak birch pollen concentrations in Central and Eastern Europe are between 550 and 1000 grains/m<sup>3</sup> [14, 23], the level of birch pollen-associated endotoxin at this time could be estimated as being 0.039–0.071 ng/m<sup>3</sup>. Castellan *et al.* [4] indicated an airborne endotoxin level of 9 ng/m<sup>3</sup> as a threshold value being associated with adverse pulmonary responses in susceptible humans exposed to cotton dust. Our calculations show that the natural endotoxin exposure related to birch pollen produces only 0.43–0.79% of the threshold. It is possible, however, that during pollination of birch, pollens of other plant species, also bearing endotoxin, are present in the air. Moreover, the concentrations of endotoxin in pollen stated in our study (3.75–37.5 ng/mg) were higher than those in house dust (0.12–20.0 ng/mg), reported by Michel *et al.* [17] as factors aggravating symptoms of house dust asthma. It is known that even in extremely small doses, endotoxin affects circulating leukocytes and platelets, activates macrophages, releases cytokines, activates the complement system, interacts with a variety of acute phase proteins, acts as a non-specific B cell mitogen, regulates the expression of Class II MHC antigens (reviewed in [2]). Endotoxin has also been shown to be a potent factor enhancing the IgE-mediated histamine release caused by specific antigens in sensitized individuals [19]. It was also suggested that endotoxin is able to eliminate the inhibitory effects of T<sub>s</sub> lymphocytes, acting thus as an adjuvant stimulating sensitization against antigens [1].

To summarize, our preliminary study suggests that the levels of endotoxin occurring in pollen are comparable to its levels in some organic dusts which are considered as potentially hazardous at work places. Cautiously stated, it cannot be excluded that in particular cases endotoxin is capable of acting as an adjuvant facilitating the initial sensitization to pollen allergens, as well as modulating the immune response to inhaled pollen grains in already sensitized subjects. A comprehensive study on this subject is in progress at our department for the possibly full assessment of the potential role of endotoxin in pollinosis.

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