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IMMUNOSTIMULATIVE EFFECTS OF REPEATED INHALATION EXPOSURE TO MICROVESICLE-BOUND ENDOTOXIN OF *PANTOEA AGGLOMERANS*

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Abstract: Rabbits exposed repeatedly to aerosols of endotoxin-containing microvesicles (ECMV) of the outer membrane of the *Pantoea agglomerans* strain isolated from airborne grain dust showed a large increase in the concentration of circulating cytokines: total interferon (IFN), interleukin-1α (IL-1 α), and tumor necrosis factor α (TNFα). The increase was significantly higher compared to animals exposed to control saline ($p<0.001$). Aerosol exposure to ECMV also induced the formation of specific precipitin antibodies and lymphocyte activation. The results indicate strong immunomodulative properties of ECMVs produced in nature by *Pantoea agglomerans* bacteria, and heavily contaminating organic dusts.

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INTRODUCTION

Gram-negative bacteria, developing on plant and animal surfaces or decomposing organic matter, produce endotoxin, a cell wall lipopolysaccharide (LPS) that is easily released into ambient air as a constituent of organic dust. Endotoxin inhaled by humans or animals causes inflammatory reactions in the lungs [6, 27]. The reactions are initiated by the activation of alveolar macrophages and other cells lining the airways, followed by the release of inflammatory mediators and an influx of neutrophils into the lung [5, 34]. Depending on the inhaled dose, endotoxin is believed to evoke organic dust toxic syndrome, chronic bronchitis, mucous membrane irritation, or to aggravate the adverse pulmonary reactions caused by exogenous allergens [16, 27].

It has been shown that the immunological and physiological response of experimental animals to the inhalation of cell-bound endotoxin (extracts of the entire cell walls of Gram-negative bacteria) was stronger compared to the purified endotoxin or the cell wall lipopolysaccharide (LPS) [22, 33]. This suggests that the most potent type of endotoxin is the naturally occurring form found in the environment. In earlier work [11, 12], we showed that environmental endotoxin found on wood dust substrates occurred in the form of globular micro-vesicles, measuring 30-50 nm, which are shed in vast amounts from the outer membranes of Gram-negative bacteria.

In order to mimic the effects of natural exposure of humans and animals to environmental endotoxin, we separated the microvesicle fraction from bacterial cells by the differential sedimentation gradients by the method

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originated by Burrell [11], and exposed laboratory animals to aerosols of this fraction in inhalation experiments. In the experiment described earlier [36], we evidenced that the long-term exposure of rabbits to aerosol of the microvesicle fraction from the cells of the *Rahnella* sp. strain isolated from airborne wood dust caused a strong specific and non-specific immunologic response.

The aim of the present study was to determine the immunologic effects of the long-term exposure of rabbits to aerosol of the microvesicle fraction from the cells of the *Pantoea agglomerans* strain isolated from airborne grain dust. *Pantoea agglomerans* (synonyms: *Erwinia herbicola*, *Enterobacter agglomerans*) is an epiphytic Gram-negative bacterium which is widespread in the agricultural working environment, and represents a significant occupational risk because of its endotoxic and allergenic properties [8, 9, 10, 21, 22].

The long-term inhalation experiments on rabbits were preceded with tests in which we compared the immunostimulative and toxic properties of the microvesicle fraction from the cells of *Pantoea agglomerans* and crude lipopolysaccharide of the same species.

MATERIAL AND METHODS

Separation of microvesicles. The *Pantoea agglomerans* strain M-10-3 isolated from the airborne grain dust in a Polish mill [10] was grown on a nutrient agar for 72 hrs at 35ºC, and the cell mass harvested with phosphate buffered saline (PBS). Endotoxin-containing microvesicles (ECMV) were separated from the *P. agglomerans* cells by differential sucrose gradients [11] using the Hoefer apparatus (Hoefer Scientific Instruments, San Francisco, CA) and lyophilized.

Comparison of the activity of ECMV and crude endotoxin. Immunostimulative and toxic properties of ECMV from the *P. agglomerans* strain M-10-3 were compared with those of the crude lipopolysaccharide (LPS-W) obtained from the cells of the same strain by the Westphal method of phenol-water extraction [39] and final lyophilization. Immunostimulative properties were tested by the examination of free cell influx to the lungs of exposed guinea pigs. Guinea pigs of either sex were divided into 3 groups, 10 animals each, for exposure to aerosols of the following substances: a) suspension of the *P. agglomerans* ECMV reconstituted in saline (0.85% NaCl) at the concentration of 1 mg/ml and sterilized by filtering through Minisart NML 0.45 µm filters; b) suspension of the *P. agglomerans* LPS-W reconstituted in saline at the concentration of 1 mg/ml and sterilized as above; c) sterile saline (0.85% NaCl) as a control. Animals of each group were exposed for 1 hr in an airtight chamber to aerosol generated by the ultrasonic Thomex L-2 nebulizer (produced in Warsaw, Poland). After 24 hrs, the guinea pigs were anesthetized by intraperitoneal injection with sodium pentobarbital and subjected to bronchoalveolar lavage (BAL) using Hanks balanced salt solution (HBSS). Total number of free alveolar cells was estimated with the use of hemacytometer and differential cell counts were made after May-Grünwald Giemsa staining.

Toxic properties were estimated by determination of LD_{50} in mice. The ECMV and LPS-W preparations were reconstituted in saline (0.85% NaCl) and sterilized by filtering through Minisart NML 0.45 µm filters. Serial dilutions were injected subcutaneously into white mice of either sex, weighing 25 g, of standard line C57BL6 obtained from the Hirszfeld Institute of Immunology and Experimental Therapy in Wrocław, Poland.

The *Limulus* activity of both preparations was determined using the Limulus Amebocyte Lysate (LAL) gel tube test (Pyroquant Diagnostik, Walldorf, Germany) according manufacturer's instructions. Commercial lipopolysaccharide *Escherichia coli* EC-5 was used as a positive control. Results were reported as endpoint doses causing coagulation (ED_{50}, ng/ml). To convert to Endotoxin Units (EU), the value in nanograms was multiplied by 10.

Long-term inhalation exposure of rabbits. Rabbits of either sex of the Polish laboratory strain "Biały Popielański" weighing 2.0–2.5 kg, were divided into 2 groups, 6 animals in each, for exposure to aerosols of the following substances: a) suspension of the endotoxin-containing microvesicles (ECMV) from the cells of the *Pantoea agglomerans* strain M-10-3, reconstituted in saline (0.85% NaCl) at the concentration of 1mg/ml and sterilized by filtering through Minisart NML 0.45 µm filters; b) sterile saline (0.85% NaCl) as a control. Rabbits belonging to each group were placed in the inhalation chamber constructed according to the design of Olenchock and Burrell [26]. A fine aerosol was generated and fed into the chamber by the ultrasonic Thomex L-2 nebulizer (produced in Warsaw, Poland). Animals were exposed 15 times, every 2nd day for 1 hr; the experiment for each group lasted therefore 1 month. It was estimated that the concentration of ECMV in the inhalation chamber was about 660 µg/m^3, conforming to the exposure in the branches of agricultural industry most at risk, such as the processing of herbs [14]. Blood samples were taken from the ear vein of each rabbit before the experiment, and after the 1st, 5th, 10th and 15th exposure. A part of each sample was used for separation of serum in order to determine the presence of specific precipitins. Heparin was added to the remaining blood in the concentration of 0.02 mg/ml, and after dispensing small volumes for *in vitro* studies, the sample was centrifuged for separation of plasma. Plasma was examined for concentrations of cytokines: total interferon (IFN), interleukin-1 α (IL-1 α) and tumor necrosis factor α (TNFα).

IFN concentration in blood plasma was determined by the method of Stewart [37] by measuring the degree of IFN-induced inhibition of the cytopathic effect caused by vesicular stomatitis virus in BF (bovine fetus cutaneous fibroblasts) tissue culture. IL-1α concentration in plasma was determined by the method of Gery *et al.* [15] in

modification of Miossec *et al.* [24] and by the MTT method in modification of Berg *et al.* [2], based on the IL-1-induced stimulation of the proliferation of mouse thymocytes (C3H line) in the presence of mitogen (phytohemagglutinin). TNFα concentration in plasma was determined by the modified method of Adams and Czuprynski [1] by measuring the degree of necrotic effect induced by this cytokine in susceptible cell culture WEHI 164 (tumor fibroblasts from mice of BALB/c line).

Phagocytic activity of granulocytes was assayed by the method of Park *et al.* [28], by adding 0.01 ml of the suspension of latex particles (∅ 0.81 µm, Difco) to 0.1 ml of blood containing 0.002 mg heparin, followed by 1 hr incubation at 37ºC, and determining the percentage of granulocytes containing at least 4 latex particles in preparations stained by the May-Grünwald method.

Induction of specific humoral immunity was assayed by the agar gel double diffusion method for the detection of precipitins against ECMV of *P. agglomerans* [29]. Induction of the specific cellular immunity was assayed by the test of the inhibition of leukocyte migration, using the capillary microcultures of whole blood after Bowszyc *et al.* [4] in modification of Milanowski *et al.* [23]. Microcultures were incubated for 4 hrs at 37ºC, with and without specific antigen (ECMV of *P. agglomerans*), at the concentration of 100 µg/ml. Results were expressed as a migration index, e.g. the ratio of the mean migration distance of leukocytes in microcultures with antigen, to the analogical distance in microcultures without antigen.

RESULTS

Comparison of the activity of ECMV and LPS-W. Both preparations were immunostimulative and caused significant influx of free alveolar cells into lungs. ECMV caused a significantly greater increase in the number of lymphocytes and neutrophils compared to LPS-W (Tab. 1). ECMV proved to be non-lethal to mice in comparison with LPS-W (Tab. 1). The activity of ECMV in the *Limulus* test was 20 times smaller compared to LPS-W.

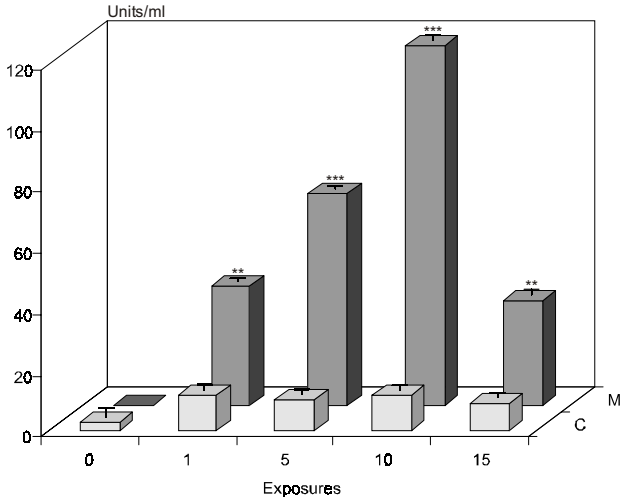

Figure 1. Concentration of total interferon in plasma of rabbits repeatedly exposed to aerosol of endotoxin-containing microvesicles of *Pantoea agglomerans*, or to aerosol of control saline. Values are geometric means (GM) ± geometric standard deviation (GSD). C - control, M – Microvesicles. *, **, *** Significantly higher than control: ** $p < 0.01$, *** $p < 0.001$.

Long-term inhalation exposure of rabbits. Long-term inhalation exposure of rabbits to *P. agglomerans* endotoxin-containing microvesicles (ECMV) caused a great and consistent increase in the levels of all the tested blood plasma cytokines: IFN, IL-1α, and TNFα. The rise in cytokine levels occurred in all exposed animals. Beginning after the first exposure, all values were significantly higher ($p<0.01$) compared to the controls (Fig. 1-3). In the blood of rabbits exposed to *P. agglomerans* ECMV, the concentrations of all cytokines were the highest in the mid-stage of the experiment, e.g. after the 5th and 10th exposures. In the final stage of the experiment (after 15 exposures), the levels of all the tested cytokines dropped markedly, but nevertheless remained significantly greater compared to controls.

No significant differences in the phagocytic activity could be found between the granulocytes from animals

Table 1. Comparison of the properties of endotoxin-contaning microvesicles (ECMV) and crude endotoxin (LPS-W).

	ECMV	LPS-W	Saline (control)
Immunostimulation			
BAL in exposed guinea pigs: total number of cells ($\times 10^6$)[a]	124.08 ± 27.92[b]	104.20 ± 23.16[b]	44.20 ±6.45
BAL in exposed guinea pigs: number of lymphocytes ($\times 10^6$)[a]	55.91 ± 13.28[bd]	33.36 ± 13.02[c]	20.26 ±4.60
BAL in exposed guinea pigs: number of neutrophils ($\times 10^6$)[a]	10.77 ± 6.39[be]	2.34 ± 0.84[b]	0.22 ± 0.22
Toxicity			
Lethality for white mice (LD_{50}, mg/kg)	>1280.0	50.0	
***Limulus* test**			
Coagulation endpoint dose (ED_{50}, ng/ml)	10.0	0.5[f]	

[a]mean ± SD; [b]significantly greater compared to control group, $p<0.001$; [c]significantly greater compared to control group, $p<0.05$; [d]significantly greater compared to guinea pigs exposed to LPS-W, $p<0.01$; [e]significantly greater compared to guinea pigs exposed to LPS-W, $p<0.001$; [f]equal to standard *E. coli* endotoxin.

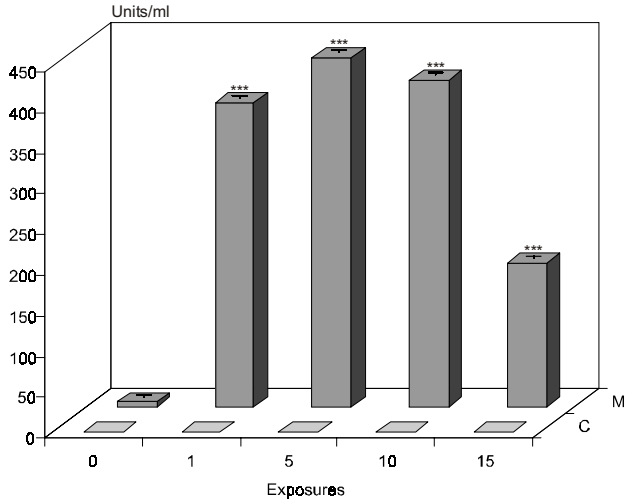

Figure 2. Concentration of interleukin-1 α (IL-1α) in plasma of rabbits repeatedly exposed to aerosol of endotoxin-containing microvesicles of *Pantoea agglomerans*, or to aerosol of control saline. Values are geometric means (GM) ± geometric standard deviation (GSD). C - control, M – Microvesicles. *, **, *** Significantly higher than control: *** $p < 0.001$.

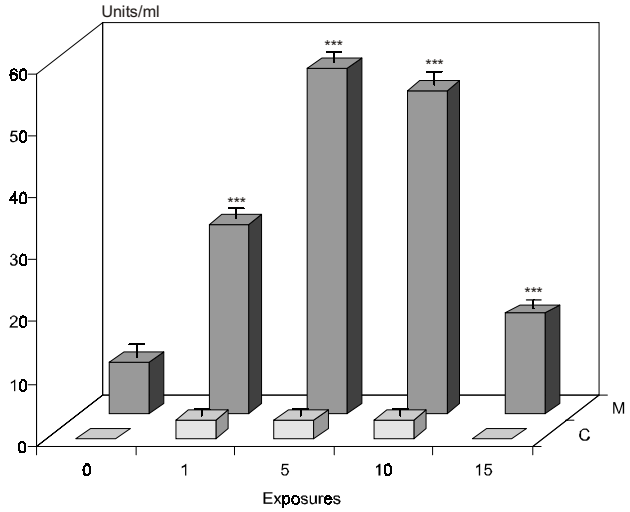

Figure 3. Concentration of tumor necrosis factor α (TNFα) in plasma of rabbits repeatedly exposed to aerosol of endotoxin-containing microvesicles of *Pantoea agglomerans*, or to aerosol of control saline. Values are geometric means (GM) ± geometric standard deviation (GSD). C - control, M – Microvesicles. *, **, *** Significantly higher than control: *** $p < 0.001$.

exposed to aerosol of *Pantoea agglomerans* ECMV, compared to those from controls (data not shown).

In the animals exposed to aerosol of *P. agglomerans* ECMV, the positive precipitin reactions to this antigen were found after 5 exposures in 2 rabbits out of 6 tested (2/6) and after 10 and 15 exposures in all rabbits tested (6/6). Precipitins were not found in the controls.

A significant drop of migration index was found in rabbits exposed to aerosol of *P. agglomerans* ECMV after 10 and 15 exposures ($p<0.001$) (Fig. 4), indicating the development of cellular immunity to this antigen after chronic respiratory exposure. The drop of migration index was not seen in controls.

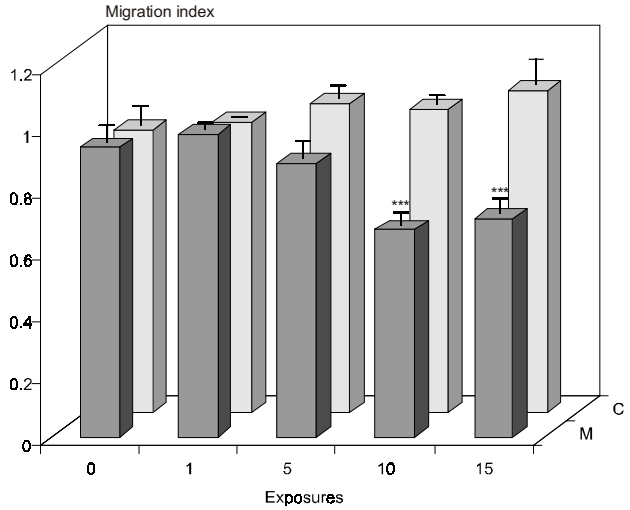

Figure 4. Inhibition of leukocyte migration at the presence of homologous antigen in the blood of rabbits repeatedly exposed to aerosol of endotoxin-containing microvesicles of *Pantoea agglomerans*, or to aerosol of control saline. Results are expressed as migration index (means ± SD). M - Microvesicles, C - control. *, **, *** Significant decrease compared to initial (0) value: *** $p < 0.001$.

DISCUSSION

The presented results confirm those of our earlier study on the effects of chronic exposure to the aerosol of the microvesicles of *Rahnella* sp. [36]. In this study, we have exposed rabbits to long-term inhalation of the microvesicles of *Pantoea agglomerans* which is probably the most common Gram-negative bacterium in the agricultural environment [9, 13], and has the ability to produce outer membrane microvesicles both on solid media and in organic dusts [12]. *Pantoea agglomerans* has been identified as a source of extremely potent endotoxin [8, 18, 19, 21, 22, 31, 35] and of allergens causing allergic alveolitis [23].

Exposure to ECMVs of *Pantoea agglomerans* caused a greater increase in the levels of blood plasma cytokines compared to ECMVs of *Rahnella* sp. Exposure to ECMVs of *Rahnella* sp. caused a significant rise in IFN level, while rises in IL-1α and TNFα were inconsistent and not significant [36]. By contrast, exposure to ECMVs of *Pantoea agglomerans* caused a significant, consistent and clear-cut rises in levels of all 3 cytokines: IFN, IL-1α, and TNFα.

The rise of the cytokines IL-1 and TNF observed in this study has been reported by earlier authors [32] after short-term inhalation exposure of guinea pigs to the *Escherichia coli* lipopolysaccharide. The interaction between endotoxin and interferon has not been fully explored, in particular in the inhalation model. Moderate stimulation of IFN production by endotoxin has been reported by earlier authors [25], but some other studies conducted on various models [3, 20] did not support these findings and the problem is still under discussion [5].

The design of our experiment conforms to the idea of Risco and Pinto da Silva [30] in that the study of effects of LPS should be conducted with preparations which resemble endotoxin in its natural environment. The results

obtained indicate that chronic inhalation exposure to endotoxin-containing microvesicles (ECMV) released from the outer membrane of Gram-negative bacteria and commonly occurring in organic dusts, evokes strong non-specific and specific immunologic responses. This suggests that the inhalation exposure of humans and animals to large amounts of ECMVs in natural environments may cause inflammatory reactions in the lungs, in particular after 5-10 exposures, when the effects are strongest.

The ECMV particle consists of lipopolysaccharide, proteins and phospholipids. Probably all these constituents, arranged in a specific conformation, jointly contribute to the strong immunotoxic effect of the ECMV "native" endotoxin, when inhaled during natural exposure.

To date, little information has been published on the chronic effects of the inhalation exposure to endotoxin [34]. The results of our examinations of the cytokine content in the blood conform to the view of earlier authors [18, 25, 34] - that in the course of the repeated exposure to endotoxin, most of its non-specific effects decrease as a result of the well-known phenomenon of endotoxin tolerance. However, a different pattern of reactivity has been observed in the development of antigen specific immunity induced by the inhaled ECMV preparations. A progressive increase with time of both antigen specific cellular and humoral responses was observed, as assayed by leukocyte migration inhibition and precipitin production, respectively. A decrease in the migration index was most probably caused by the cytokine IL-4 (migration inhibition factor) released by the specific reaction between repeatedly inhaled antigen and sensitized T-lymphocytes.

Both the ECMVs and crude endotoxin of *P. agglomerans* showed the immunostimulative properties causing the influx of free alveolar cells into lungs of exposed guinea pigs, described by earlier authors [31, 35]. In contrast to endotoxin, EMCVs proved to be non-toxic for mice, and showed lower activity in *Limulus* test.

In conclusion, our results clearly demonstrate that the microvesicle form of *Pantoea agglomerans* endotoxin commonly occurring in nature, strongly stimulates cytokine production. This fact could be associated with health risks, considering the possibility of inflammatory reaction and adverse effects of IFN acting synergistically with TNF and endotoxin [7], but on the other hand, particularly at lower exposure, may have beneficial effects. The anti-cancer properties of IFN and other cytokines are well known [38], and the enhancement of its level by repeated, usually occupational, exposure to the ECMV inhalation, might explain, at least in part, the cancer-protective effects of environmental endotoxin suggested by Rylander [32, 34] and Lange *et al.* [17]. Accordingly, our results indicate the need for experimental studies on the usefulness of endotoxin-containing microvesicles of *Pantoea agglomerans* in immunotherapy of cancer. The important argument for the therapeutic use of these microvesicles is that they proved to be non-toxic for warm-blooded animals.

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