MOUSE MODEL OF HYPERSENSITIVITY PNEUMONITIS
AFTER INHALATION EXPOSURE TO DIFFERENT MICROBIAL ANTIGENS
ASSOCIATED WITH ORGANIC DUSTS

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Abstract: The aim of this study was to reproduce in laboratory conditions hypersensitivity pneumonitis (HP) pathogenesis in a new animal model predictive of the human response, and to select the microbial antigen associated with organic dust that exerts the strongest pathogenic effect on the respiratory organ. To achieve this goal, mice of the strain C57BL/6J prone to fibrosis were exposed for 1 hour daily up to 28 days to the inhalation of aerosols of each of the 5 microbial components of organic dusts whose conjunction with the occurrence of HP has been confirmed by numerous authors: *Pantoea agglomerans* saline extract (SE), *P. agglomerans* microvesicle-bound endotoxin, *Saccharopolyspora rectivirgula* SE, *Aspergillus fumigatus* SE, saline extract of dust from a grain sample overgrown with *S. rectivirgula* and *Thermoactinomyces vulgaris*, and a saline solvent (PBS) was used as a control. Exposure of the animals to organic dust components was conducted using a novel inhalation challenge set. Lung samples were collected from untreated mice and from mice exposed for 7 and 28 days, and examined by digitalized histopathology and biochemistry for the presence of inflammatory changes and fibrosis. *P. agglomerans* SE appeared to be the sole antigen which evoked a statistically significant fibrosis and a significant increase of hydroxyproline in the lungs of mice exposed for 28 days to this extract, both compared to the mice untreated and to those exposed to the solvent. *P. agglomerans* SE also evoked the strongest and statistically significant inflammatory response in the lungs of the mice, both after 7 and 28 days of exposure. After 7 days, significant inflammatory changes were also found in mice exposed to *A. fumigatus* SE, and after 28 days in mice exposed to all antigens. In conclusion, our results allow us to define a useful animal model of HP which can be a supplement for now commonly used bleomycin model. This model should comprise: present set of instruments for inhalation, mice of the line C57BL/6J and the saline extract of *P. agglomerans* as the antigen. For a better understanding of the presented results, a detailed study covering immunological investigations, focused on the mechanism of antigen action, are needed.

INTRODUCTION

Hypersensitivity pneumonitis (HP), or extrinsic allergic alveolitis, is a granulomatous interstitial disease of the lungs due to immune reactions following chronic inhalation of finely dispersed organic dusts or chemicals, especially encountered in the occupational environment. Th1 T lymphocytes and alveolar macrophages play a central role in the pathogenesis of the disease. Recurrent acute, subacute, and chronic HP may either progress to irreversible

lung fibrosis or provoke emphysematous changes [13, 31, 32, 38, 60, 62, 66, 74, 76, 82, 84, 85].

HP comprises a number of variants (farmer's lung, thresher's lung, grain handler's disease, suberosis, wood chips disease, malt fever, bagassosis, bird fancier's lung and many others) which show a similar clinical course, but are caused by different offending dusts containing a variety of specific etiological factors, including: thermophilic actinomycetes, fungi, Gram-negative bacteria and bird proteins [1, 5, 6, 8, 20, 21, 29, 36, 43, 45, 48, 57, 61, 64, 72]. The pathogenic agents are mostly glycoproteins produced by dust-borne fungi and bacteria, less often animal and plant proteins [28, 44].

To elucidate the pathogenic pathways of HP, numerous animal experiments have been conducted with the use of various antigens, exposure techniques and animal species (guinea pigs, golden hamsters, rabbits, mice, rats) [12, 30, 33, 42, 46, 77]. These studies made a significant progress in understanding the pathogenesis of HP; however, some pathways and their genetic backgrounds remain unclear. Accordingly, the aim of this study was to reproduce HP in laboratory conditions in a new animal model predictive of the human response, and to select the microbial antigens associated with organic dust that exert the strongest pathogenic effect on respiratory organ. To achieve this goal, mice of the strain C57BL/6J prone to fibrosis [14] were chronically exposed to finely dispersed aerosols of each of 5 microbial components of organic dusts, whose conjunction to the occurrence of HP has been confirmed by numerous authors, including our group [18, 34, 36, 40, 44, 45, 57, 61].

The components include: 1–2) Two antigens of thermophilic actinomycetes: • saline extract (SE) of the cells of *Saccharopolyspora rectivirgula* (synonyms: *Faenia rectivirgula*, *Micropolyspora faeni*) and • saline extract (SE) of the dust from grain sample overgrown with *S. rectivirgula* and *Thermoactinomyces vulgaris*. Both species develop in moist, overheated hay and other plant materials, and their etiological role in causing farmer's lung in subjects exposed to these materials has been documented by numerous authors [9, 29, 43, 61, 64].

3) One antigen of mould fungi: • saline extract (SE) of the mycelium of *Aspergillus fumigatus*. Mould fungi, especially those belonging to the genera *Aspergillus* and *Penicillium*, are the frequent cause of HP cases following exposure to mouldy wood, grain and other materials [1, 37, 45, 63, 67]. *A. fumigatus* was described as a causative agent in the disease of paper factory workers exposed to mouldy beech chips [20, 59] and in other forms of HP [44, 45].

4–5) Two antigens of the Gram-negative bacterium *Pantoea agglomerans* (synonyms: *Erwinia herbicola*, *Enterobacter agglomerans*) were used: • saline extract (SE) of the cells and • microvesicle-bound endotoxin (MV-LPS), occurring abundantly in organic dusts [25]. *P. agglomerans* is widely distributed on plant and plant materials and reveals strong allergenic and endotoxic properties [16, 17, 18, 23, 26, 53, 54,

68, 69, 73]. This bacterium was identified as an important etiological factor of HP among agricultural workers in Poland, especially those handling grain [21, 41, 49, 51, 57].

MATERIALS AND METHODS

Animals

3-month-old female C57BL/6J mice were purchased from Charles River Laboratories, GmbH, Germany and used in the study. The mice were fed a standard diet and water *ad libitum* and housed under standard conditions. The experimental protocol was approved by the Local Bioethics Committee in Lublin, Poland.

Antigens

5 antigens used in the study were prepared as follows:

Saline extract (SE) of the cells of *Saccharopolyspora rectivirgula*. Standard strain A-94 obtained from the Rothamsted Experimental Station, UK, was inoculated on enriched nutrient agar medium (BTL, Łódź, Poland) supplemented with peptides (Proteobak, BTL, Łódź, Poland). Cultures were incubated in Roux bottles for 72 hrs at 55°C. The bacterial mass was harvested by washing off with sterile distilled water, homogenized with glass homogenizer and extracted in saline (0.85% NaCl) in the proportion 1:2 for 48 hrs at 4°C, with intermittent disruption of cells by 10-fold freezing and thawing. Afterwards, the supernatant was separated by centrifugation at 10,000 rpm at 4°C and finally lyophilized.

Saline extract (SE) of the dust from grain overgrown with thermophilic actinomycetes. 420 grams of barley grain in an Erlenmayer flask was inoculated with the suspensions of the cells of *Saccharopolyspora rectivirgula* (strain A-94, as above) and *Thermoactinomyces vulgaris* (strain EW-23 isolated from the air of grain store) in 0.85% NaCl, each in the volume of 25 ml containing 10^6 cells/ml. Inoculated grain was incubated for 1 month at 55°C. Afterwards, the concentrations of *S. rectivirgula* and *Th. vulgaris* on grain were determined by dilution plating as 7.0 × 10^6/g and 3.7 × 10^6/g, respectively. Next, 200 grams of infected barley was extracted in saline (0.85% NaCl) in the proportion of 1:10 for 72 hrs at 4°C, with intermittent shaking 7 times per day. Afterwards, the extraction fluid was filtered through gauze and subjected to 10-fold freezing and thawing. Then, the supernatant was separated by centrifugation at 10,000 rpm at 4°C, and finally lyophilized.

Saline extract (SE) of the mycelium of *Aspergillus fumigatus*. Standard strain IG-1 obtained from the Institute of Tuberculosis and Pulmonary Diseases in Warsaw was inoculated on nutrient broth (BTL) supplemented with 4% glucose. Cultures were incubated in Erlenmayer flasks for

21 days at 30°C. Superficial mycelium was suspended in culture broth and homogenized with a laboratory mixer. Next, the cell suspension was extracted in saline (0.85% NaCl) in the proportion 1:2 for 48 hrs at 4°C, with intermittent disruption of cells by 10-fold freezing and thawing. Afterwards, the supernatant was separated by centrifugation, dialysed against tap water for 48 hrs, and then against distilled water for 24 hrs, and finally lyophilized.

Saline extract (SE) of the cells of *Pantoea agglomerans*. The strain M-10-3 isolated from the air of a grain mill, and used for over 30 years as a standard strain in the research conducted by the researchers of the Institute of Agricultural Medicine in Lublin [18, 19, 42, 50, 56, 77], was inoculated on enriched nutrient agar medium (BTL, Łódź, Poland) supplemented with peptides (Proteobak, BTL, Łódź, Poland). Cultures were incubated in Roux bottles for 72 hrs at 37°C. Further steps of antigen preparation (harvesting of bacterial cell mass, homogenization, extraction, disruption by freezing and thawing, separation of supernatant, lyophilisation) were the same as in the preparation of the extract of *Saccharopolyspora rectivirgula*.

Endotoxin-containing microvesicles (MV-LPS) of *Pantoea agglomerans*. MV-LPS was isolated as described earlier [24, 27, 81]. Briefly, the strain M-10-3 of *P. agglomerans* was grown on a enriched nutrient agar (BTL) for 72 hrs at 35ºC, and the cell mass was harvested with phosphate buffered saline (PBS, Biomed). Endotoxin-containing microvesicles were separated from the *P. agglomerans* cells by differential sucrose gradients using the Hoefer apparatus (Hoefer Scientific Instruments, San Francisco, CA) and lyophilized.

Long-term inhalation exposure of mice

Six lots of the C57BL/6J 3-months-old female mice, each of 16 animals, were examined before treatment (4 animals) while the remaining 12 animals were exposed for 28 days, 1 hour daily, to finely dispersed aerosols of the following substances: a) saline extract of the *Saccharopolyspora rectivirgula* cell mass, b) saline extract of barley grain overgrown with thermophilic actinomycetes, c) saline extract of *Aspergillus fumigatus* mycelium, d) saline extract of *Pantoea agglomerans* cell mass, e) endotoxin-containing microvesicles of *Pantoea agglomerans*, f) sterile PBS (Biomed) as a control. For each inhalation experiment, the lyophilized extracts of agents (a–e) were dissolved in PBS in the concentration of 1 mg/ml, except for saline extract of the dust from grain overgrown with thermophilic actinomycetes, which was dissolved in PBS in the concentration of 10 mg/ml.

Animals exposure to organic dust components was conducted using a novel inhalation challenge set constructed according to own design [35], and consisted of an ultrasonic aerosol generator, airtight chamber with 15 perforated containers for the mice, and a vacuum pump. This set assured

a continuous flow of a fine droplet aerosol composed of particles measuring on the average 1.77 µm, which could easily penetrate into the deep parts of the lungs, alveoli and bronchioli. The concentration of the aerosolized extracts in the chamber, measured by weighing filters mounted at the outlet of chamber before and after exposure, was in the range ($\bar{x} \pm SD$): $35.6 \pm 10.7 - 112.8 \pm 50.3$ µg/m^3.

Before treatment and after 7 and 28 days of exposure, the lung and blood samples were taken from 4, 6, and 6 mice, respectively. Sera were separated from the blood samples by centrifugation and tested with the antigens used in the inhalation exposure experiments. The lung incision samples were kept at room temperature and at -20°C, and sent, respectively, to the University of Verona, Italy, for histological examination and to the Université Catholique de Louvain, Brussels, Belgium for determination of hydroxyproline level.

Agar-gel precipitation test

The agar-gel precipitation test was performed by Ouchterlony double diffusion method in purified 1.5% Difco agar (Detroit, MI, USA) supplemented with 0.1% sodium azide with the antigens (saline extracts) of *S. rectivirgula*, *A. fumigatus*, *P. agglomerans* and grain dust overgrown with thermophilic actinomycetes. Because of the low yield of blood collected from experimental animals, the obtained serum samples were pooled before the use in assay. Serum was placed in the central well and antigens, dissolved in 0.85% NaCl at the concentration of 30 mg/ml, in the peripheral wells. The plates were incubated for 6 days at room temperature. Then, preliminary examination was made for the development of characteristic precipitation lines. After reading, the plates were washed in saline supplemented with 0.1% sodium azide for 4–5 days, and then in 5% sodium citrate solution for 90 min (to prevent false positive reactions). Next, the plates were stained with azocarmine B and a final reading was made [65, 78, 79].

Histological examination – H&E and Masson trichrome stainings

The lung tissue was cut into sections 2×5 mm thick and fixed in 4% buffered formalin for 12 hrs, followed by dehydration in ascending series of alcohol and embedding in paraffin wax. 5 µm thick sections were obtained from the paraffin blocks and stained with hematoxylin and eosin (H&E) or Masson trichrome. Micrographs were evaluated by light microscopy to assess their general morphology (H&E staining) and to detect collagen fibres (Masson trichrome staining). The histological examination was performed by a pathologist, who was blinded to the experimental protocol, using an image analysis system (D-Sight system, Menarini Diagnostics, Florence, Italy).

Lung injury was scored according to the following 4 aspects: centrolobular inflammation, interstitial inflammation,
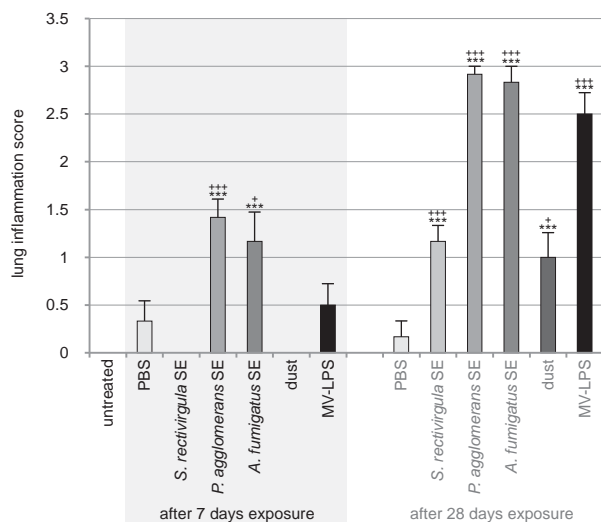
Figure 1. Quantification of inflammation in untreated mice (control) and animals exposed to organic dust components and PBS through 7 or 28 days. Data for histologic scores are given as mean ± SEM of investigated items. *** at least p<0.001 *vs.* control; + at least p<0.05 *vs.* PBS samples; +++ at least p<0.001 *vs.* PBS samples; one-way ANOVA test; post test: Tukey; dust = grain dust containing thermophilic actinomycetes (SE), MV-LPS = endotoxin-containing microvesicles of *P. agglomerans*.
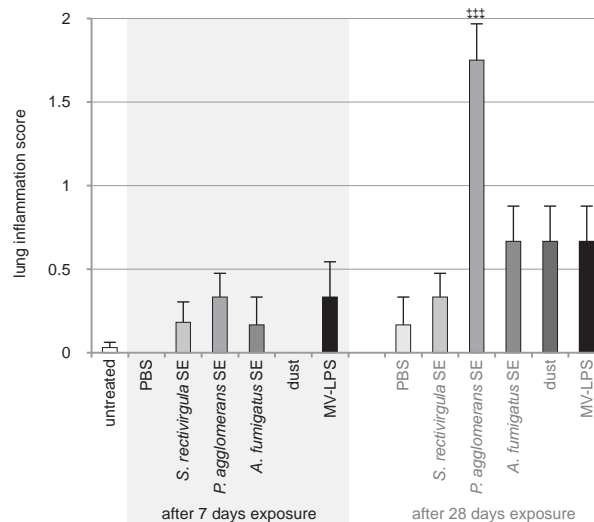
peribronchial fibrosis and interstitial fibrosis. These features were graded with 4 point scales: 0 = regular tissue, 1 = mild changes, 2 = moderate changes, 3 = significant changes. Individual parameters: lung inflammation score and lung fibrosis score were calculated separately as the mean of investigated items in every research group.

Biochemical examination – hydroxyproline level determination

Lavaged lungs were perfused via the right heart ventricle with saline, excised and placed into a Falcon tube chilled on ice. Two ml of 0.9% saline were added and the lungs were then homogenized with an Ultra-Turrax T25 homogenizer (Janke and Kunkel, Brussels, Belgium) for 30 seconds and stored at -80°C for later use. Collagen deposition was estimated by measuring the hydroxyproline contents of lung homogenates by high-performance liquid chromatography (HPLC) as previously described [3].

Statistical analyses

Statistical analyses were performed by GraphPad Prism 5. Data obtained from histological examination were presented as the mean value and standard error of the mean (SEM). Data obtained from hydroxyproline assay were presented as the median value, 25–75% percentile and range. In both cases, the experimental groups were compared to saline-exposed mice and untreated animals. For assessment of histological and biochemical data, statistical analysis was performed using the one way-ANOVA with Tukey post-hoc test. Significance was accepted at p<0.05.

Figure 2. Quantification of fibrosis in untreated mice (control) and animals exposed to organic dust components and PBS through 7 or 28 days. Data for histologic scores are given as mean ± SEM of investigated items. *** at least p<0.001 *vs.* control; +++ at least p<0.001 *vs.* PBS samples; one-way ANOVA test; post test: Tukey; dust = grain dust containing thermophilic actinomycetes (SE); MV-LPS = endotoxin-containing microvesicles of *P. agglomerans*.

RESULTS

Effectiveness of inhalation exposure – serological testing. It was found that only the mice exposed for 28 days to *P. agglomerans* SE produced specific precipitating antibodies against inhalation factor. All other results were negative, including serological response of mice against homologous antigens after 7 and 28 days, and the response of mice untreated and treated with saline against all tested antigens.

Histological examination – H&E and Masson trichrome staining. Statistically significant signs of inflammation were observed after 7 and 28 days of inhalation exposure to saline extracts of *P. agglomerans* and *A. fumigatus* (Fig. 1). Observed changes were significant compared to both untreated and control (treated with solvent without organic dust) animals. After 7 and 28 days of inhalation exposure to *P. agglomerans* and *A. fumigatus*, inflammation scores for these antigens were, respectively, 1.417/2.917 and 1.167/2.833 (Fig. 1). The exposure of mice to other organic dust components induced statistically significant lung tissue damage only after 28 days of exposure. Inflammation changes observed after 28 days of exposure were significant in mice exposed to all examined organic dust components, compared to untreated animals and to animals treated with solvent without organic dust. Inflammation scores for *S. rectivirgula*, grain dust and *P. agglomerans* MV-LPS were 1.167, 1.0 and 2.5, respectively.

Statistically significant lung fibrosis was observed after 28 days of inhalation exposure to *P. agglomerans* saline extract (Fig. 2). Fibrosis score for this harmful agent was
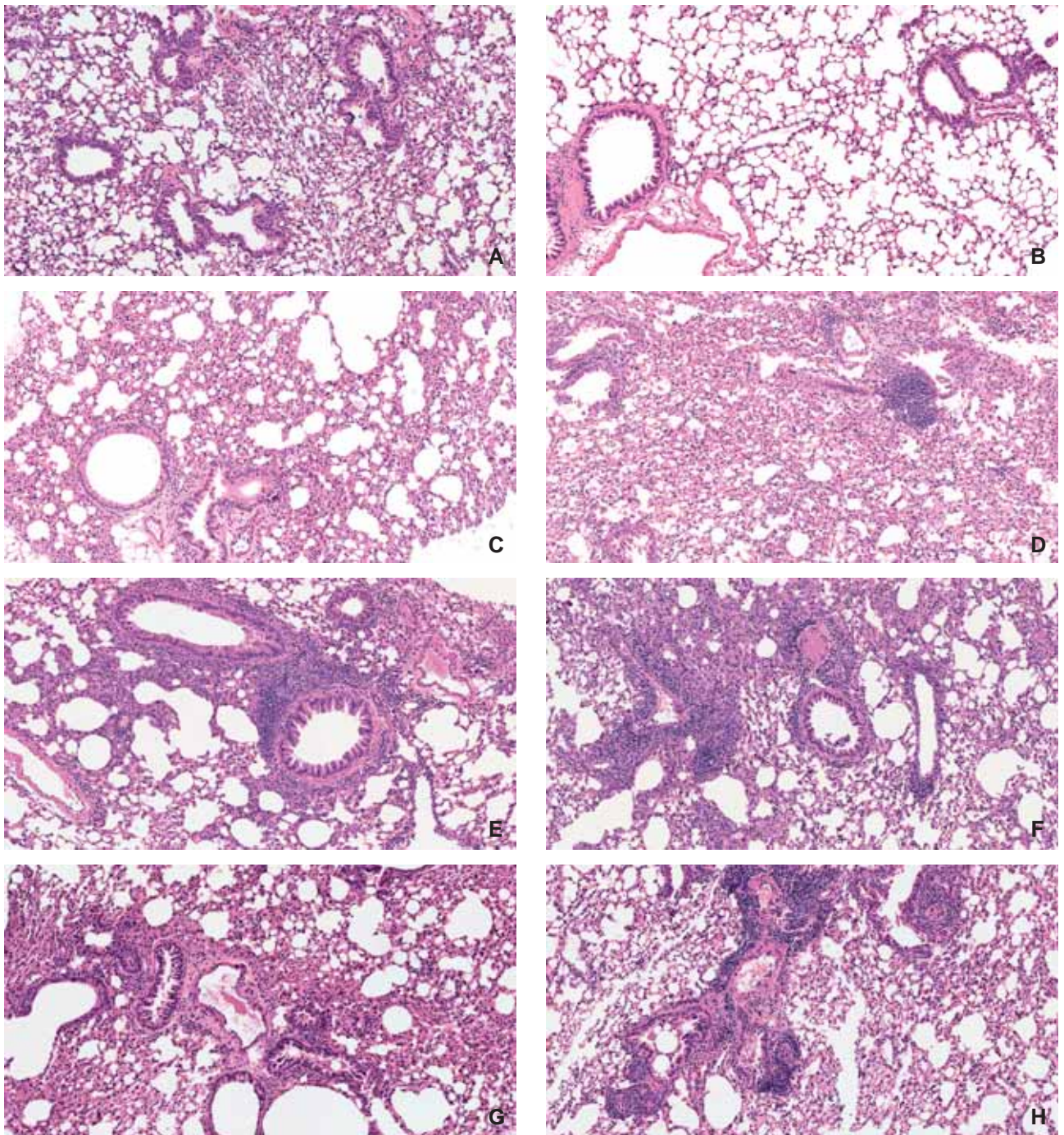
Figure 3. Histopathology examination of lungs after hematoxylin and eosin staining of mice exposed to PBS (control) (A, B) and organic dust components: *S. rectivirgula* SE (C, D); *P. agglomerans* SE (E, F); *A. fumigatus* SE (G, H). Photographs A, C, E, G present the results after 7 days of animals exposure to investigated factor. Photographs B, D, F, H present the results after 28 days of exposure. Data shown represent 8 mice from 64 separate experiments for H&E staining.

1.75 and was significantly greater compared to both untreated and control (treated with solvent without organic dust) animals (p<0.001). The exposure of mice to other organic dust components did not provoke statistically significant signs of fibrosis.

The effect of mice exposure to organic dusts components on lung morphology was assessed using light microscopy after hematoxylin-eosin staining (Figures: 3A–H, 4I–L).

After mice exposure to saline (PBS) there was no inflammatory response noted (Figures: 3A, 3B). The same effect was observed after 7 days of mice exposure to *S. rectivirgula*; although after 28 days of exposure signs of inflammation were increasing (Figures: 3C, 3D). On the contrary, significant centrolobular and interstitial inflammation with marked infiltration of lymphocytes were observed after 7 days of exposure to *P. agglomerans*. Additionally, alveolar

distortion and thickening of the alveolar walls were observed in this investigated group. After 28 days of exposure, the inflammatory process grew more intense. Respiratory ducts showed almost complete obstruction of lumen due to lymphocyte and macrophage infiltration (Figures: 3E, 3F). Lung response to *A. fumigatus* after 7 and 28 days of exposure gave almost the same picture as observed in *P. agglomerans* group (Figures: 3G, 3H). The histological picture of samples obtained from animals exposed to grain dust containing thermophilic actinomycetes was the same as observed in the group of animals exposed to saline, but in "time point 28" signs of inflammation increased (Figures: 4I, 4J). 7 days of mice inhalation exposure to endotoxin-containing microvesicles of *P. agglomerans* provoked a weak inflammatory response, but after 28 days of treatment the inflammatory response grew more intense. Changes observed in this investigated group were similar in terms of their nature to those observed in samples from mice exposed to *P. agglomerans* saline extract, but the effect was not so strong and significant (Figures: 4K, 4L).

To detect collagen fibres, lungs collected from mice exposed to organic dusts components were subjected to Masson trichrome staining. Increased collagen levels were only detected in mice exposed for 28 days to *P. agglomerans*. In all other exposed mice there were no deviations from the norm. Changes in the group of mice exposed to *P. agglomerans* are presented in Figures 6B–D.

Biochemical examination – hydroxyproline level determination. Amplitude of the pulmonary fibrosis induced
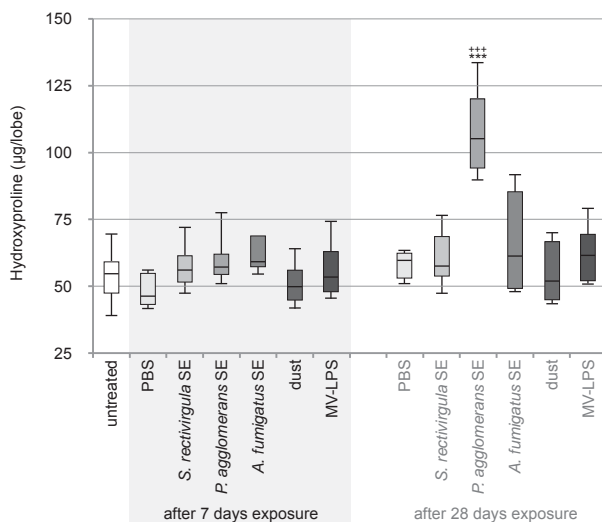
Figure 5. Concentration of hydroxyproline in lung homogenates received from untreated mice (control) and animals exposed to organic dust components and PBS through 7 or 28 days. Results are expressed as: median of hydroxyproline concentration in investigated lobe, 25-75% percentile and range (0-100% percentile). *** at least p<0.001 *vs.* control; +++ at least p<0.001 *vs.* PBS samples; one-way ANOVA test; post test: Tukey; dust = grain dust containing thermophilic actinomycetes (SE); MV-LPS = endotoxin-containing microvesicles of *P. agglomerans*.

by chronic exposure to organic dusts was determined by measuring the hydroxyproline level. The values of hydroxyproline concentration in lung homogenates of mice exposed to organic dust components are presented in Figure 5. After 7 days of exposure, none of assessed agents caused
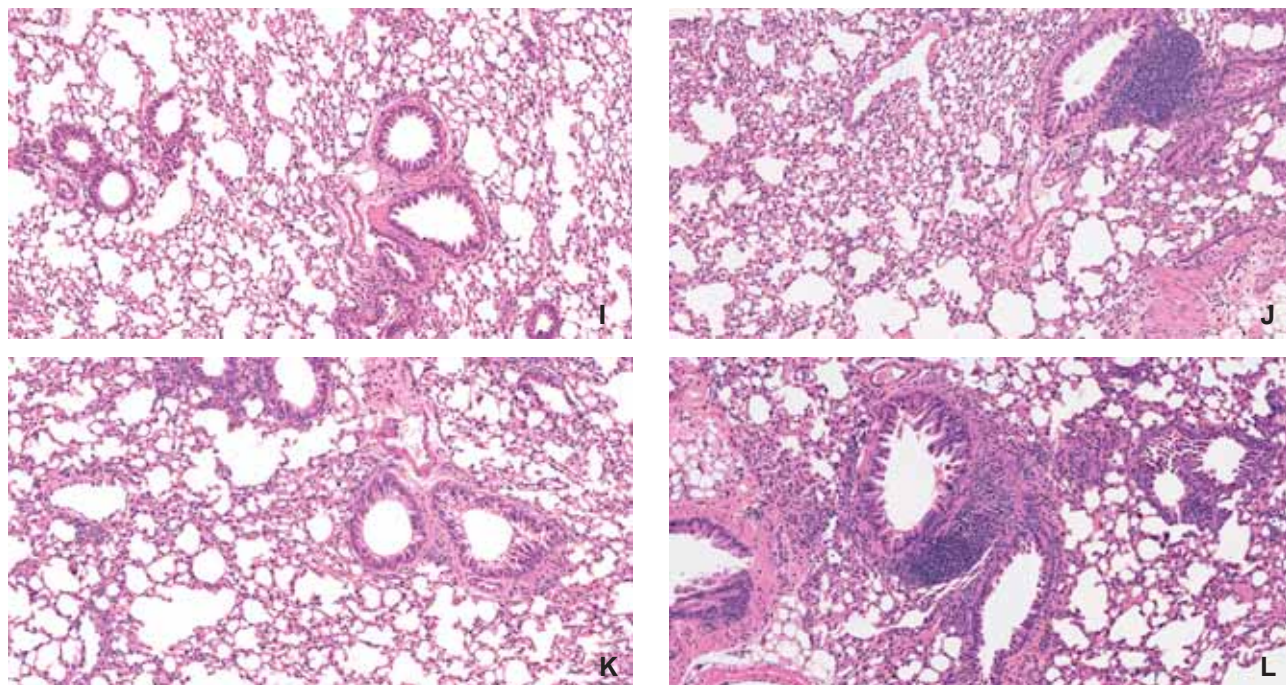
Figure 4. Histopathology examination of lungs after hematoxylin and eosin staining of mice exposed to grain dust SE (I, J); MV-LPS of *P. agglomerans* (K, L). Photographs I, K present the results after 7 days of animals exposure to investigated factor. Photographs J, L present the results after 28 days of exposure. Data shown represent 4 mice from 32 separate experiments for H&E staining.
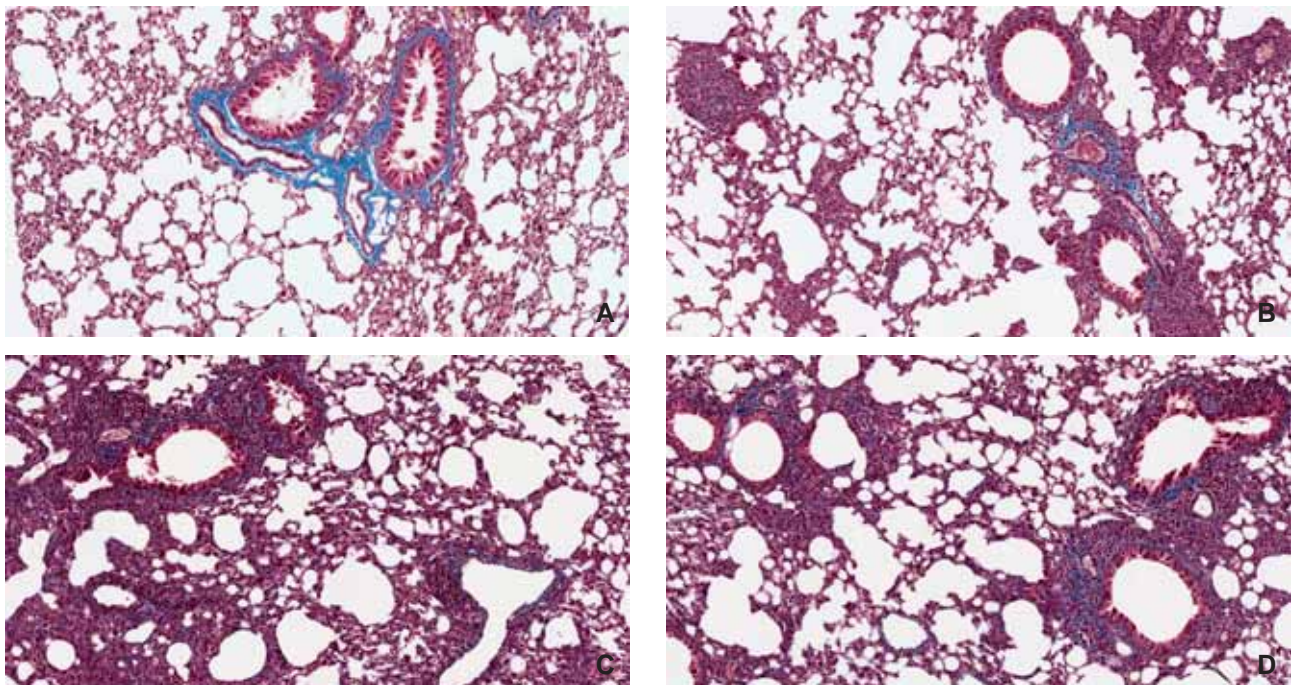
Figure 6. Histopathology examination of lungs after Masson trichrome staining from the mice untreated (A) and exposed to *P. agglomerans* (SE) for 7 days (B) or 28 days (C, D). Data shown represent 4 mice from 32 separate experiments for Masson trichrome staining.

a statistically significant difference in hydroxyproline concentration compared to untreated samples or saline samples. After 28 days of exposure, only in the samples from mice exposed to *P. agglomerans* (saline extract) a clear-cut and statistically significant (p<0.001) increase of hydroxyproline level compared to both untreated and saline samples was detected.

DISCUSSION

An essential step in the development of an animal model for hypersensitivity pneumonitis (HP) is the standardisation of a reliable exposure system. In recent experimental studies of murine HP, the intranasal or intratracheal instillation of the pathogenic factors were mostly utilised [12, 75, 83]. These systems allow for exact measurements of given doses but do not mimic the real exposure existing in the environment. Aiming to overcome this disadvantage, we designed and created a new mice inhalation system, whose operating principle is based on a natural process of acquiring the harmful agent by breathing a fine aerosol dispersed in the ambient air. This method assures also the penetration of allergen into the deep parts of lungs, alveoli and bronchioli, which is important from the standpoint of HP disease. Another advantage of our system is the possibility to determine the dose of agent needed for inducing desired changes [35].

The results of this study show that our model allows the reproduction, in controlled laboratory conditions, of the circumstances in which a HP occurs. The use of the saline extract of the Gram-negative bacterium *Pantoea agglomerans* evoked

both acute (short period of exposure amounted to 1 week; predominant inflammation) and chronic (long period of exposure amounted to 4 weeks; predominant fibrosis) histopathological changes in the lungs of exposed mice. The character of these changes was similar to those recorded by Lantz *et al.* [46] in hamsters exposed to *P. agglomerans* endotoxin and to those recorded by Kuś *et al.* [42] in guinea pigs exposed to saline extract of *P. agglomerans*. In the present study, the saline extract of *Pantoea agglomerans* caused a significant growth of the inflammation score, both after 1-week and 4-week exposure (p<0.001, both *versus* untreated and PBS-treated controls), which was the strongest compared to all other antigens (Fig. 2). Moreover, the *P. agglomerans* SE was the only one of all the investigated antigens that caused a significant increase of fibrosis score after 4-week exposure (p<0.001) (Figures: 3, 6). This growth was supported biochemically by the significant increase of hydroxyproline level at the same time interval (p<0.001), that was found only in the mice exposed to *P. agglomerans* SE (Fig. 5).

The histopathological changes caused by the environmental, globular endotoxin produced by *Pantoea agglomerans* (referred to as endotoxin-containing microvesicles, microvesicle-bound endotoxin or MV-LPS) were much weaker compared to the saline extract of this bacterium. After 1-week exposure, the *P. agglomerans* MV-LPS caused only a slight increase in the inflammation score that became distinctly significant after 4-weeks exposure (p<0.001), although lower than *P. agglomerans* SE.

The saline extract of *Aspergillus fumigatus* was the second after *P. agglomerans* SE with respect to the growth of

the inflammation score. It caused the significant increase of this score both after 1-week and 4-weeks exposure (Fig. 1). However, this antigen did not cause a significant increase in the fibrosis score and hydroxyproline level, similar to all the antigens, except *P. agglomerans* SE.

The saline extracts of *Saccharopolyspora rectivirgula* and of grain dust containing thermophilic actinomycetes caused a significant increase in the inflammation score after 4-weeks exposure. This growth, however, was weaker compared to *P. agglomerans* SE, *P. agglomerans* MV-LPS and *A. fumigatus* SE (Fig. 1). These results, together with the lack of fibrotic changes and of an increased hydroxyproline level, suggest that *S. rectivirgula* and/or other thermophilic actinomycetes are not useful factors for developing an experimental HP in the mouse model.

Pantoea agglomerans (synonyms: *Erwinia herbicola*, *Enterobacter agglomerans*) is a fermentative, epiphytic bacterium widely distributed in nature, especially on the surface of plants. It constitutes the prevailing component of the microflora of grain, cotton, herbs, flax and many other plant materials used in industry [10, 11, 16, 22, 70]. *P. agglomerans* predominates also in inhalable dust from grain, herbs, and flax, and was identified as a premier cause of allergy in grain workers, herb workers and flax farmers [17, 18, 23, 26, 34, 39, 78-80]. It has been documented that this bacterium is the most important cause of HP in eastern Poland [21, 41, 49, 51, 57]. Besides allergenic properties, *P. agglomerans* is a source of an extremely potent endotoxin [16, 46, 47, 53, 54, 68, 73] that could be a potential cause of lung inflammation and organic dust toxic syndrome (ODTS, toxic pneumonitis) in workers exposed to grain dust or other dusts of plant origin. It has been demonstrated that environmental endotoxin produced by *P. agglomerans* occurs in dust in the form of globular vesicles measuring 30-50 nm (referred to as endotoxin-containing microvesicles, microvesicle-bound endotoxin or MV-LPS) which are composed of lipopolysaccharides, proteins and phospholipids [24, 25]. In the inhalation experiments conducted on rabbits, the microvesicle-bound endotoxin showed a potent biological activity causing significant increase of circulating cytokines, formation of specific precipitins and lymphocyte activation, although its *Limulus* activity amounts to only 5% of chemically isolated endotoxin [27]. These results were confirmed by another study in which MV-LPS caused a significant increase in cytokine production by cultures of human peripheral blood mononuclear cells [81]. The fact that the dust-borne endotoxin of *Pantoea agglomerans* occurs in the form of fine, submicroscopic microvesicles easily penetrating into alveoli, increases the potential risk of disorders after exposure to these bacteria.

The results presented in this study indicate that cell extract of *P. agglomerans* causes pulmonary changes the characteristic of HP, which are similar to but stronger compared to those caused by MV-LPS. These results are in line with those obtained by Burrell and Ye [7] who reported a significantly greater influx of free cells into the lungs

of hamsters exposed to aerosol of whole cells of *P. agglomerans,* compared with those exposed to homologous endotoxin. They conform also to the results obtained by Rylander [71] who found that the cell-bound endotoxin of *P. agglomerans* showed a stronger effect on the increase of free macrophages and lymphocytes in the lungs of guinea pigs, and a decrease in respiratory function in humans (drop of FEV_1) than isolated endotoxin of this species. Similarly, in the experimental research conducted by researchers from the Lublin centre, with the use of *P. agglomerans* saline extract prepared in the same way and from the same strain as in the present study, this extract showed a potent biological activity that was stronger or equal to the activity revealed by *P. agglomerans* endotoxin obtained by the Boivin trichloroacetic acid extraction [19, 42, 50, 52, 53, 54, 55, 56, 77]. Milanowski [54] found in the inhalation experiments on guinea pigs that saline extract of *P. agglomerans* caused a significantly greater increase in the number of free alveolar cells, of superoxide anion production by lung cells, and of the breathing rate, than did the homologous endotoxin. Similarly, Dutkiewicz and Kuś [19] found a potent effect of the saline extract of *P. agglomerans* on the influx of free cells into the lungs of guinea pigs, and Kuś *et al.* [42] demonstrated interstitial inflammatory lesions and fibrosis of the interalveolar septa in the lungs of guinea pigs exposed for a long time to this extract.

The reason for the more potent biological activity of the cell extract of *P. agglomerans* compared to endotoxin produced by this species is unknown. Endotoxin by itself cannot be the main cause of saline extract activity as it amounts to only 1% of *Limulus* activity recorded for isolated endotoxin [23]. One of the possibilities is the presence of a strong protein antigen in the cell wall of *P. agglomerans*, comparable to superantigens in other bacteria, that could synergize with endotoxin present in this wall and exert strong biological effects [2, 4]. However, this assumption must be verified by further research. Nevertheless, our results clearly demonstrate that *P. agglomerans* commonly occurring in nature, has the ability to provoke in mice C57BL potent changes typical of hypersensitivity pneumonitis after chronic exposure. These results also suggest that *P. agglomerans* is one of the most important agents in the agricultural environment as far as occupational disease is concerned.

We demonstrated that also other antigens used in this study, in particular *Aspergillus fumigatus*, the common fungus described as a cause of HP, APE (asthma with pulmonary eosinophilia) and occupational aspergillosis of the lungs [15, 20, 58, 59], after long-lasting exposure could evoke a significant inflammatory reaction in lungs, but their effects are much weaker than those caused by *P. agglomerans* and do not include fibrosis of the lung tissue.

In conclusion, our results allow us to define a useful animal model of HP which can be a supplement for the now commonly used bleomycin model. This model should comprise: present set of instruments for inhalation, mice of

the line C57BL/6J, and saline extract of *P. agglomerans* as the antigen. Nevertheless, for a better understanding of the presented results, a detailed study covering immunological investigations, focused on understanding the mechanism of antigen action, are needed. For now, our results are promising and encourage further research.

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