ALLERGIC ALVEOLITIS AMONG AGRICULTURAL WORKERS IN EASTERN POLAND: A STUDY OF TWENTY CASES

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Abstract: The aim of this study was to identify the specific agents which caused extrinsic allergic alveolitis (EAA) in the selected group of 20 agricultural workers from eastern Poland. The microbiological analysis of the samples of plant materials or dusts reported by the patients as causing symptoms has been carried out, followed by allergological tests (inhalation challenge, agar-gel precipitation test, inhibition of leukocyte migration, skin test) with extrinsic microbial antigens. It was found that the causative agents of allergic alveolitis in the examined group of patients were mesophilic, non-branching bacteria associated with grain dust, mostly *Pantoea agglomerans* (synonyms: *Erwinia herbicola, Enterobacter agglomerans*) and *Arthrobacter globiformis* (each in eight cases). The remaining agents were *Alcaligenes faecalis* (in two cases), and *Brevibacterium linens* and *Staphylococcus epidermidis* (in one case each). On the basis of the clinical picture, the bronchoalveolar lavage (BAL) and allergological tests, the diagnosis of the chronic form of the disease was stated in 14 patients and an acute form - in 6 patients. EAA patients demonstrated in the BAL fluid a typical lymphocytic alveolitis both in terms of percentage and absolute number of lymphocytes. Also, the numbers of eosinophils and neutrophils were significantly higher in EAA patients.

INTRODUCTION

Extrinsic allergic alveolitis (hypersensitivity pneumonitis, granulomatous pneumonitis) is a T-lymphocyte dependent granulomatous inflammatory reaction, predominantly of the peripheral gas exchange tissue of the lung [29, 37, 53, 55]. It comprises many forms (subunits) caused by a range of bioaerosols associated with a particular type of occupational exposure to organic dust (farmer's lung, malt fever, suberosis, bagassosis, mushroom grower's disease, bird fancier's lung and others) [16]. The pathogenic allergens are mostly glycoproteins produced by dust-borne fungi and bacteria, less often animal and plant proteins [14, 28]. Hence, most of the causative agents of the disease were identified by the microbiological examination of the working environment, including processed materials, dust, air, waste and soil [1, 20, 25, 28, 39, 47]. The identification of the etiological agent is of basic importance for the diagnostics, treatment and prevention of the disease [11, 55].

Table 1. Examined group of patients with diagnosis of allergic alveolitis.

No. Initials		Gender	Age	Smoking	Profession	Material reported to cause symptoms
1.	WZB	M	47	No	Grain elevator worker	Barley
2.	RE	F	32	No	"	"
3.	RK	M	45	No	"	"
4.	WJ	M	52	No	"	"
5.	MM	M	48	No	"	"
6.	SZS	M	50	No	"	"
7.	BJ	M	43	No	"	"
8.	WZD	M	59	Yes	"	"
9.	SZ	F	46	No	Farmer	"
10.	KD	F	50	No	"	"
11.	NL	F	55	No	"	"
12.	RW	F	61	No	"	"
13.	KAL	F	58	No	"	Mixed grain
14.	ŁE	M	32	No	Animal feed facility worker	"
15.	MA	M	66	Yes	Grain store worker	"
16.	KK	M	19	Yes	Farmer	Clover
17.	KAN	M	28	No	"	"
18.	SS	M	30	Yes	"	"
19.	SJ	M	29	No	"	"
20.	KS	M	35	No	"	Hay + barley

The most common form of allergic alveolitis among agricultural workers is farmer's lung, the disease originally described by Campbell [4]. Pepys *et al.* [47] have proved, on the basis of microbiological and serological examination of the samples of mouldy hay associated with the disease cases, that the most common cause of farmer's lung in England are thermophilic actinomycetes of the species *Saccharopolyspora rectivirgula* (synonyms: *Faenia rectivirgula, Micropolyspora faeni, Thermomonospora monospora*) developing in moist, overheated hay and other plant materials. This finding has been confirmed in other countries [6, 15, 28, 40] and therefore the antigens of *S. rectivirgula* are widely used for the immunological diagnostics of allergic alveolitis in agricultural workers.

However, it appeared from further microbiological studies of offending dusts that besides *S. rectivirgula*, other actinomycetes and fungi, such as *Thermoactinomyces vulgaris, Aspergillus flavus* and *Aspergillus versicolor* may cause farmer's lung or similar work-related forms of allergic alveolitis in agricultural workers [26, 45, 52]. It

was found that etiological agents of the disease in various countries may differ depending on climatic conditions, type of vegetation and other physiographical factors [11]. Terho [58] and Terho and Lacey [59] examined a large number of fodder samples associated with cases of farmer's lung in Finland and found that the fungus *Aspergillus rubrobrunneus* (synonym: *Aspergillus umbrosus*, the anamorph of *Eurotium rubrum*) was the most common cause of the disease in that country. The studies carried out by Kuś [22, 23] and others [10] suggest that the Gram-negative bacteria *Pantoea agglomerans* (synonyms: *Erwinia herbicola, Enterobacter agglomerans*), associated with grain, are an important etiological factor of allergic alveolitis among agricultural workers in Poland.

The aim of this study was to attempt to identify the agents causing allergic alveolitis in a selected group of agricultural patients from eastern Poland with the use of microbiological and immunological methods. In addition, clinical features of the disease were characterised. Preliminary results of this study have been reported elsewhere [34, 35, 36].

MATERIALS AND METHODS

Patients

Twenty patients with the diagnosis of allergic alveolitis were examined. This group consisted of 14 males and 6 females aged 19–66 yrs, mean 41.3 yrs. Four persons were smokers, the others never smoked cigarettes. Ten patients were farmers, eight worked in a big grain elevator, one in a small grain store and one in an animal feed producing facility. All the patients had been exposed during work to the inhalation of organic dusts of plant origin. In 15 patients the disease symptoms were caused by grain dust, in four by dust from clover and in one by dust from hay (Tab. 1).

The patients were selected for this study in two ways:

1) Eleven patients were selected out of the group of 17 agricultural patients of the Clinic of Lung Diseases of the Medical Academy in Lublin with diagnosis of allergic alveolitis. Six patients did not take all the tests and were not included in the study group.

2) Nine patients were selected out of the groups of 80 workers of a big grain elevator and 30 workers of an animal feed producing facility who were subjected to detailed medical examinations. Allergic alveolits was diagnosed in eight workers of the grain elevator (10.0%) and one worker of the animal feed facility (3.3%).

Samples of offending materials

Thirteen samples of plant materials and dusts reported by the patients as causing symptoms were collected in sterile Erlenmayer flasks for microbiological analysis (Tab. 2). The number of samples was smaller than the number of patients, as two groups of patients (Nos. 1–8

Table 2. Examined samples of offending materials causing symptoms in patients.

No.	Material	Initials of patients reporting this material as causing symptoms
1.	Barley dust from elevator	WZB, RE, RK, WJ, MM, SZS, BJ, WZD
2.	Barley grain from elevator	"
3.	Barley grain from barn	SZ
4.	"	KD
5.	"	NL
6.	"	RW
7.	Mixed grain from barn (barley + rye + oat)	KAL
8.	Mixed grain from animal feed production facility (barley + oat + corn)	ŁE
9.	Grain dust from animal feed production facility	"
10.	Mixed grain from store (barley + rye + oat)	MA
11.	Dust from clover collected in barn	KK, KAN, SS, SJ
12.	Hay from barn	KS
13.	Dust from barley collected in barn	KS

and Nos. 16–19) reported the same materials as causing symptoms (barley + barley dust and dust from clover, respectively). On the other hand, patients Nos. 14 and 20 reported two materials each as causing symptoms and both these materials were examined (Tab. 2). As seen in Table 2, in 11 out of 13 samples the offending material was grain or grain dust (mostly from barley) and in the remaining two cases it was dust from clover and hay.

Microbiological examination

The concentration and species composition of bacteria and fungi in the collected samples was determined by dilution plating. One gram of each sample was suspended in 100 ml of sterile saline (0.85% NaCl) containing 0.1% (v/v) of Tween 80 and after vigorous shaking, serial 10-fold dilutions in saline were made up to 10^{-10}. The 0.1 ml aliquots of each dilution were spread on duplicate sets of the following media:

1) Blood agar plates for estimation of the total mesophilic bacteria;

2) Half-strength tryptic soya agar (*Difco*) plates for estimation of thermophilic actinomycetes;

3) Malt agar (*Difco*) plates for estimation of fungi.

The blood agar plates were subsequently incubated for one day at 37°C, 3 days at 22°C and 3 days at 4°C. The tryptic soya agar plates were incubated for 5 days at 55°C. The malt agar plates were subsequently incubated for 4 days at 30°C and 4 days at 22°C [8]. The grown colonies were counted and differentiated and the data were reported as cfu (colony forming units) per gram of the sample. The total concentration of microorganisms per gram of sample was obtained by the addition of the concentrations of mesophilic bacteria, thermophilic actinomycetes and fungi.

Bacterial isolates were identified with microscopic and biochemical methods, as recommended by Bergey's Manual [21, 57, 58] and Cowan & Steel [5]. Fungi were classified with microscopic methods, according to Barron [2], Litvinov [32], Ramirez [50], and Raper & Fennell [51]. The percent species composition of the total microflora (mesophilic bacteria + thermophilic actinomycetes + fungi) was then determined. The microbial species which reached the highest percent value in the total microflora of the sample was defined as "dominant microorganism".

Electron microscopy. Ultrastructural examination of the bacterial mass of coryneform isolates was done as described earlier [12]. Briefly, small portions of bacterial mass collected from agar slant cultures were pre-fixed in 2% glutaraldehyde in phosphate buffer at pH 7.3, and post-fixed in 1% buffered osmium tetroxide. After dehydration in graded series of ethanol, the samples were embedded in Low Viscosity (by dr Spurr), thin sectioned (silver colour) and post-stained with 2% uranyl acetate and lead citrate. The micrographs were taken with a Philips EM 300 electron microscope operating at 80 KV.

Allergological tests

All the patients were tested by agar-gel precipitation test, test for the inhibition of leukocyte migration (MIF), skin test and inhalation challenge. Each patient was tested in all tests with three antigens:

1) antigen prepared from the microorganism dominant in his/her own sample of offending material causing disease symptoms;

2) antigen prepared from the A-94 strain of *Saccharopolyspora rectivirgula* received from dr J. Lacey (IACR Rothamsted, Harpenden, UK) as the factor widely recognised as the most important cause of allergic alveolitis in agricultural population [17, 28, 46];

3) antigen prepared from the M-10-3 strain of *Pantoea agglomerans,* isolated from the air of a rye mill in Poland and routinely used for preparing antigenic extracts in the Institute of Agricultural Medicine in Lublin [9], as the agent suggested as important in causing allergic alveolitis in Poland [10, 23, 24, 35, 36].

Antigens for all the tests were prepared according to the unified procedure described below and used in different concentrations, depending on the test. All antigens were produced in the Institute of Agricultural Medicine in Lublin.

Preparation of antigens. In all tests lyophilised saline extracts of bacterial mass were used. In the case of *Pantoea*

agglomerans and other mesophilic, non-branching bacteria the mass was harvested from nutrient agar cultures which had been incubated in Roux bottles at 34°C for 48 hrs. The mass was then 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 distilled water for 24 hrs, concentrated by evaporation to 0.1–0.15 of previous volume and lyophilised.

The mass of *Saccharopolyspora rectivirgula* was harvested from sugar broth cultures which had been incubated in Erlenmayer flasks at 50°C for 4 weeks, homogenised and further extracted and processed as above.

Agar-gel precipitation test. The test was performed by Ouchterlony double diffusion method in purified 1.5% *Difco* agar. The patient's serum was placed in the central well and antigens, at the concentration of 30 mg/ml, in the peripheral wells. Each serum was tested twice: not concentrated, and 3-fold concentrated, for the detection of low levels of precipitins. The plates were incubated for 6 days at room temperature, then washed in saline and in 5% sodium citrate solution (to prevent false positive reactions), and stained with azocarmine B [48].

Test for inhibition of leukocyte migration in the presence of specific antigen. The test was performed by the whole blood capillary microculture method according to Bowszyc *et al.* [3]. Patient's blood and Parker's culture medium was added in the volumes of 0.5 ml and 0.12 ml, respectively, to two silicon test tubes. Then, 0.12 ml of the antigen solution in the concentration of 25 µg/ml was added to one tube, while to the other 0.12 ml of the diluent (P.B.S.) as a control. Both suspensions were incubated for 30 min at room temperature and thereafter distributed to heparinised glass capillars 75 × 1 mm. Capillars were sealed at both ends with a 4 : 1 mixture of paraffin and vaseline, centrifuged for 10 min at 1500 rpm and fastened tangentially on microscopic slides with sticky tape at an angle of 10°. The microcultures thus obtained were incubated for 4 hrs at 37°C in a humid chamber. The leukocyte migration distances, visible as distinct white zones, were measured under the binocular microscope. The results were expressed as a migration index (MI), e.g. the ratio of the mean migration distance of leukocytes in microcultures with antigen, to the analogical distance in microcultures without antigen. The test was considered as positive at the MI equal to 0.790 or lower.

Skin test. The antigens were dissolved in 0.85% NaCl at a concentration of 1 mg/ml, sterilised by filtering and checked for sterility and lack of toxicity. The test was performed by intracutaneous injection 0.1 ml of the antigenic extracts and of saline (as a control) into the forearm of the patient. The test sites were observed after 20 min for immediate reactions, after 8 hrs for delayed reactions and after 24 hrs for late reactions. The wheal and/or erythema reactions of 5 mm or more in diameter (at negative control) were regarded as positive.

Inhalation challenge. All patients were informed in detail about the aim and possible effects of the test and gave consent for its performance. The antigens were dissolved in 0.85% NaCl in a very low concentration of 20 µg/ml, sterilised and checked as above. The antigenic solutions were administered to patients by the TUR-USI-3 ultrasonic nebulizer (produced in Germany) for a period of 3 minutes. The tests with the allergens of *Saccharopolyspora rectivirgula*, *Pantoea agglomerans* and dominant microorganism (if other than *P. agglomerans*) were preceded with the control test with saline (0.85% NaCl) used for the measurement of diurnal variability of spirometric values. At each test, spirometric measurements were performed before the test (at 8 a.m.) and after 2 min, 30 min, 4 hrs, 8 hrs and 24 hrs post inhalation challenge. They comprised determination of vital capacity (VC) and forced expiratory volume in one second (FEV$_1$). The decrease of VC and/or FEV$_1$ by 10% of the initial value was considered as a positive result of the test. The patients were observed through 48 hrs post challenge for the appearance of general and respiratory symptoms. The body temperature was measured 8 hrs after challenge. Blood sedimentation, number of leukocytes in peripheral blood and serum immunoglobulins (IgA, IgG, IgM) were determined before and 24 hrs after test.

Clinical examinations

Routine clinical examination comprised: 1) detailed anamnesis considering the time - relationship of the symptoms and exposure to organic dust, 2) physical examination, 3) laboratory examination (morphological and biochemical blood tests, IgA, IgG, IgM levels), 4) chest X-ray, 5) spirometric measurements: vital capacity (VC) and forced expiratory volume in one second (FEV$_1$).

Bronchoalveolar lavage (BAL) was performed as described by Hunninghake *et al.* [18]. Briefly, 50 ml of physiological saline was introduced into the segmental bronchus of the right middle lobe through a fiberoptic bronchoscope, followed by suction at low pressure. The procedure was repeated three times. Approximately 65% of the instilled fluid was recovered. The cells were separated from the lavage fluid by centrifugation at 400 g for 10 min, washed with Hank's solution, and counted microscopically using a Bürker chamber. The composition of the BAL cells was estimated by cytocentrifuged smears using May-Grünwald-Giemsa stain. Cell viability was monitored by counting the percentage of cells which excluded 0.4% trypan blue dye. A total of 12 patients were examined by this method. The BAL results in the examined patients were related to the results achieved in the control group of 17 healthy

persons (13 males + 4 females, mean age 41.8 ± 13.0 years) without exposure to organic dusts [49].

Statistical analysis

The significance of the differences between the results of allergological tests with different allergens and between the results of the analysis of BAL fluid in the groups of EAA patients and control subjects was assessed by the use of Student's t-test.

RESULTS

Microflora of dust samples. Results of the microbiological examination of the samples of dust and plant materials reported by patients as causes of symptoms, are shown in Fig. 1. The total concentration of viable microorganisms in the examined samples was within the range 10^5–10^{10} cfu/gram. In all samples clearly dominated non-branching mesophilic bacteria (mainly Gram-negative rods, coryneform bacteria, staphylococci) while the percentages of actinomycetes and fungi were low, and never exceeded 9% and 26%, respectively.

Gram-negative bacteria were the most numerous microbial group in eight out of 13 samples. In seven samples they made up over 55% and in four samples over 75% of total viable microorganisms. The species *Pantoea agglomerans* was identified as a dominant microorganism in five samples (four of grain and one of clover dust), whereas *Alcaligenes faecalis* in three samples (two of barley and one of hay). Coryneform bacteria dominated in three samples, forming 70–97.3% of the total count. In two samples of barley and barley dust from a grain

elevator a dominant species was *Arthrobacter globiformis* (Fig. 11–12), which constituted over 90% of the total count. In another sample of barley grain, the dominant species was *Brevibacterium linens*. The species *Staphylococcus epidermidis* was a dominant microorganism in two samples of mixed grain and grain dust from an animal feed producing plant.

Response rates of the patients to the extracts of environmental microorganisms. The overall results of the tests are shown in Fig. 2. In most cases patients responded with the greatest rate to the extracts of dominant microorganisms. This is best seen in the results of the inhalation challenge read 4, 8 and 24 hrs post exposure. In all these cases the response to the extracts of dominant microorganisms was significantly greater compared to *S. rectivirgula* and *P. agglomerans* (p < 0.05), and the difference was most distinct 8 hrs post exposure (p < 0.001 and p < 0.01, respectively).

The skin response of patients to the extracts of dominant microorganisms was at all time intervals (20 min, 8 hrs and 24 hrs) significantly greater compared to *S. rectivirgula* (p < 0.05), but not to *P. agglomerans*. Precipitin reactions to *S. rectivirgula* occurred significantly less often (p < 0.05) compared to dominant microorganisms (with concentrated sera) and *P. agglomerans* (both with concentrated and not concentrated sera). No significant differences in the response to particular extracts were found in the MIF test.

The results of the tests, in particular of the inhalation challenge, enabled us to identify the dominant microorganisms as the primary etiological agents of the disease in all twenty patients: *Pantoea agglomerans* in
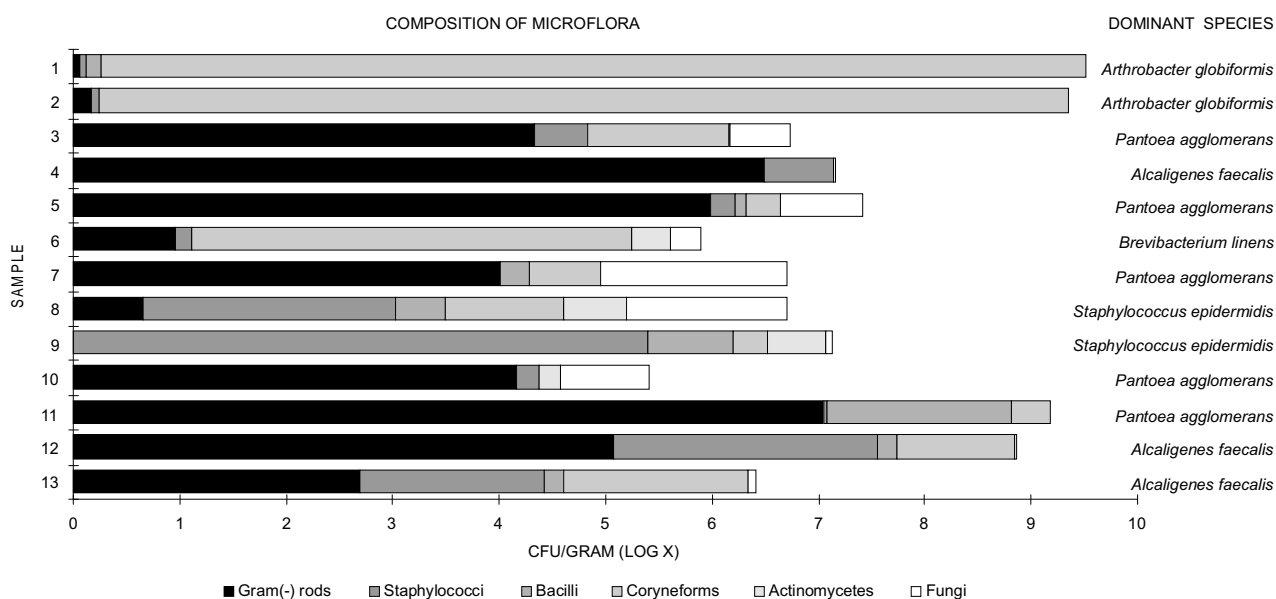

Figure 1. The microflora of samples of plant materials and dusts causing symptoms in the patients. The numbers of the samples correspond to those in Table 1. Lengths of the bars show total concentration of microorganisms (cfu/gram) in logarithmic values. The segmentation of bars presents proportional composition of the microflora. Dominant species for each sample are listed on the right.

Table 3. Results of clinical examination of EAA patients.

No. Initials	Symptoms	Aus.	Chest X-ray	Spiro-metry	Diagno-sis
1. WZB	SB, C, F, CP, WL	CR	ID	N	CEAA
2. RE	SB, C, F, CP, GM, WL	N	N	N	,,
3. RK	SB, C, F, CP, GM, WL	CR	ID	N	,,
4. WJ	SB, C, F, CP, GM	CR	ID	N	,,
5. MM	SB, C, F, CP	DBF	ID, E	N	,,
6. SZS	SB, C, F, CP, GM	CR	ID	M	,,
7. BJ	SB, C, CP, GM, WL	DBF	ID	R	,,
8. WZD	SB, C, CP, H, GM	CR	ID	N	,,
9. SZ	SB, C, CP, GM	N	ID	N	,,
10. KD	SB, C, CP, GM, H, WL	CR	ID, F, E	M	,,
11. NL	SB, C, CP, GM, H, WL	CR	ID, F	M	,,
12. RW	SB, C, CP, GM, H, WL	CR	ID, F	R	,,
13. KAL	SB, C, CP, GM	CR	ID	R	,,
14. ŁE	SB, C, CP, GM, WL	CR	ID	R	AEAA
15. MA	SB, C, GM, WL	CR	ID	N	CEAA
16. KK	SB, C, F, CP, GM	CR	ID	N	AEAA
17. KAN	SB, C, F, GM, WL	CR	ID	N	,,
18. SS	SB, C, F, GM, WL	CR	ID, F	N	,,
19. SJ	SB, C, F, H	CR	ID	N	,,
20. KS	SB, C, CP, GM, H	W	ID	N	,,

Explanation: Symptoms: SB - shortness of breath, C - cough, F - fever, CP - chest pain, GM - general malaise, H - haemoptysis, WL- weight loss; Auscultation (Aus.): CR - crackles, DBF - decreased breathing sounds, W - wheezing, N - normal; Chest X-ray: ID - interstitial diffuse changes, F - fibrosis, E - emphysema, N - Normal; Spirometry: N - normal, R - restriction type impairment, M - mixed type impairment; Diagnosis: CEAA - chronic form of EAA, AEAA - acute form of EAA.
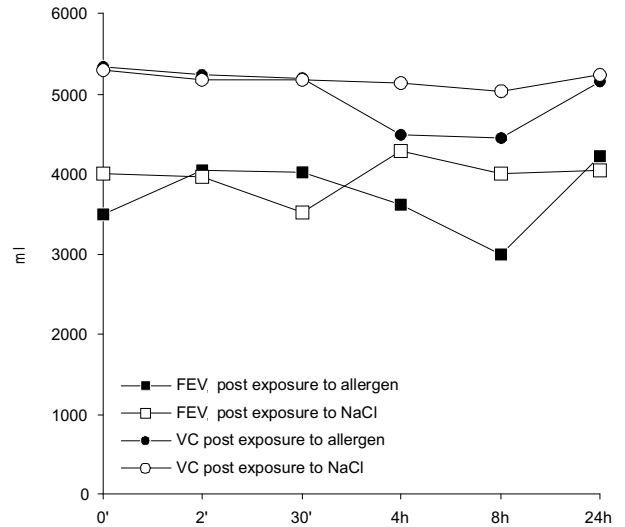

Figure 3. Results of inhalation challenge with the extract of dominant microorganism (*Pantoea agglomerans*) in patient S.S. Note drop of spirometric values 4-8 hrs after the inhalation.

eight, *Arthrobacter globiformis* in eight, *Alcaligenes faecalis* in two, *Brevibacterium linens* in one, and *Staphylococcus epidermidis* in one.

Characteristics of the response to inhalation challenge. Three main types of the response in respiratory function were found, expressed by decrease of FEV_1 after exposure to bacterial extracts. The most common type characterised itself by a single rapid decrease after 4–8 hrs post exposure, followed by a rise at 24 hrs post exposure (Fig. 3). The second type was a "dual" reaction characterised by the the immediate decrease after 2–30 min, followed by the second drop after 8–24 hrs (Fig. 4). The third type manifested itself as a steady decrease from 2 min until 24 hrs post exposure (Fig. 5). The response in VC in most
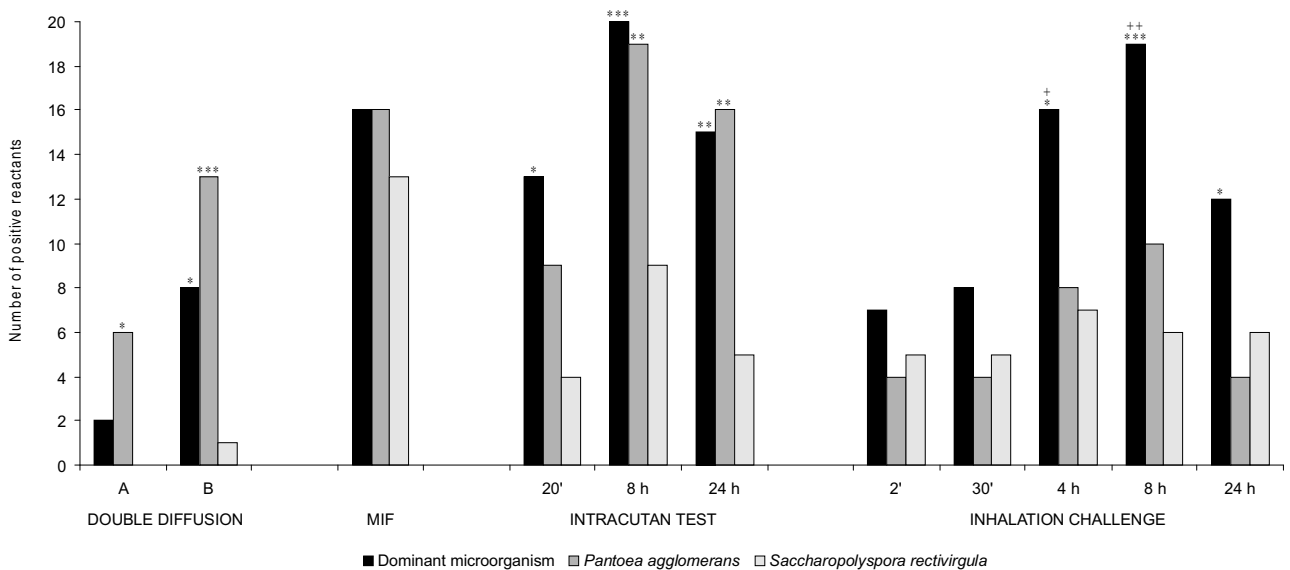

Figure 2. Results of allergological tests in the examined group of patients. MIF = test for inhibition of leukocyte migration in the presence of specific allergen. * - *** = significantly greater compared to *S. rectivirgula*, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. + - ++ = significantly greater compared to *P. agglomerans*, + $p < 0.05$, ++ $p < 0.01$.
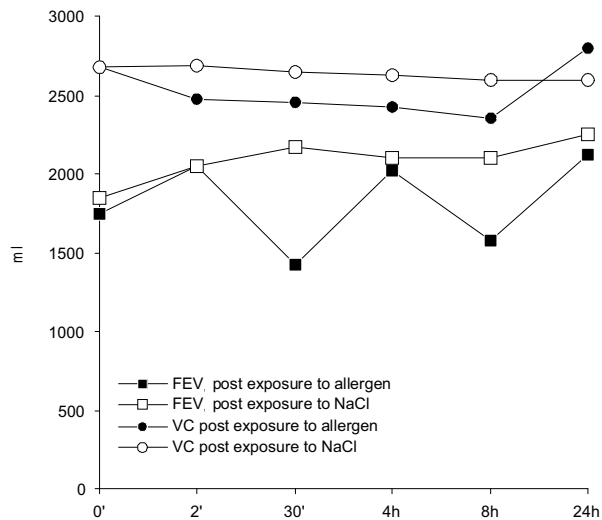
Figure 4. Results of inhalation challenge with the extract of *Pantoea agglomerans* in patient R.E. Note "dual" type response.
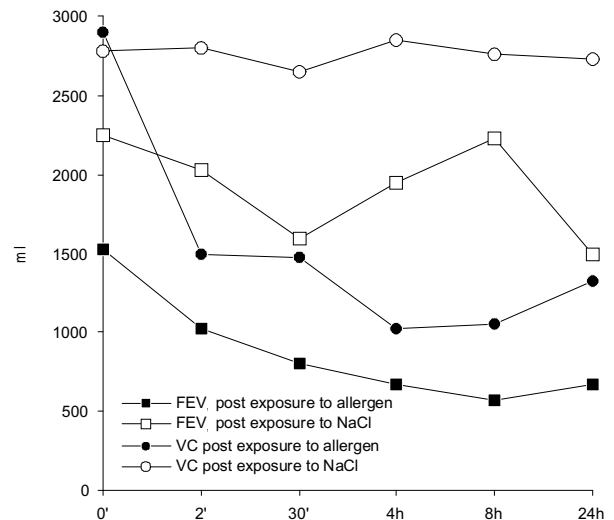

Figure 5. Results of inhalation challenge with the extract of dominant microorganism (*Staphylococcus epidermidis*) in patient Ł.E. Note slow decrease of spirometric values during 24 hrs after the provocation.

cases characterised itself by a single, distinct decrease 4–8 hrs post exposure (Fig. 3–5).

After inhalation challenge with the extracts of dominant microorganisms, there was observed a significant increase in the number of leukocytes in peripheral blood of the patients ($p < 0.01$) and a not significant ($p > 0.05$) increase in the levels of all examined classes of immunoglobulins (IgG, IgA, IgM) (Fig. 6). Twelve out of 20 patients exposed to dominant microorganisms responded with general and respiratory symptoms (muscle aches, malaise, headache, chest tightness) and seven patients showed a rise of body temperature by 1.3–2.4°C. All these symptoms were transient and in none of the patients was a harmful effect of the inhalation challenge observed.

Clinical characteristics of the patients. The results of clinical examination are shown in Table 3. In all 20 EAA patients the symptoms were compatible with allergic alveolitis clinical picture: they always occurred 4–8 hours after exposure to organic dust. The frequency of symptoms (in descending order) were: dry cough (in all 20 patients), shortness of breath (20), general malaise (17), chest pain (15), weight loss (11), fever (9), haemoptysis (6).

Physical examination by auscultation found inspiratory crackles in 15 patients, decreased breathing sounds in two patients and wheezing in one patient. In two patients the results of physical examination were normal.

In chest X-ray, interstitial micronodular and reticular changes were found in 19 patients. They occurred mostly in lower and middle lung fields (Fig. 7–8). Pulmonary fibrosis was found in four patients (Fig. 9) and emphysematous changes in two patients (Fig. 10). In one patient chest X-ray was normal.

Pulmonary function tests showed the restrictive type impairment in four patients, mixed-type in three patients and normal spirometry in the remaining 13 patients.

The morphological and biochemical blood tests did not show significant abnormalities.

On the basis of clinical picture, the diagnosis of the chronic form of the disease was stated in 14 patients and an acute form in 6 patients.

As presented in Table 4, the amounts of lavage fluid recovered from patients with EAA by BAL were comparable to those observed in control subjects. By contrast, EAA patients demonstrated a statistically significant increase of cell recovery compared to control subjects ($p < 0.001$). EAA patients demonstrated a typical lymphocytic alveolitis both in terms of percentage and absolute number of lymphocytes which was 16 times
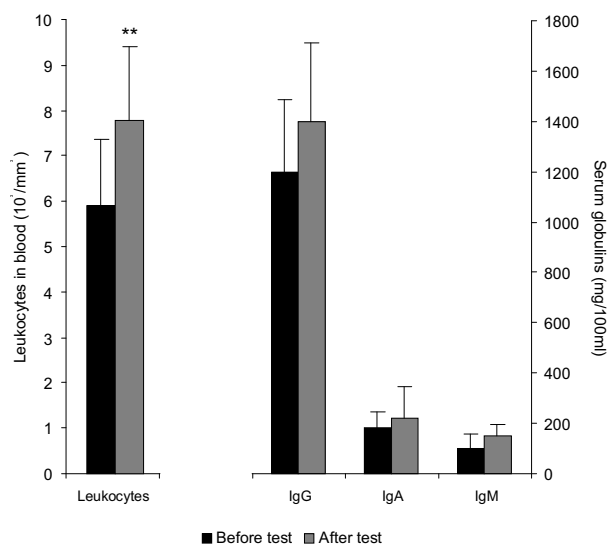

Figure 6. Numbers of leukocytes in peripheral blood and concentrations of immunoglobulins in serum of patients before and 24 hrs post inhalation challenge with the extracts of dominant microorganisms ($\bar{x} \pm$ S.D.). ** Significant increase ($p < 0.01$).
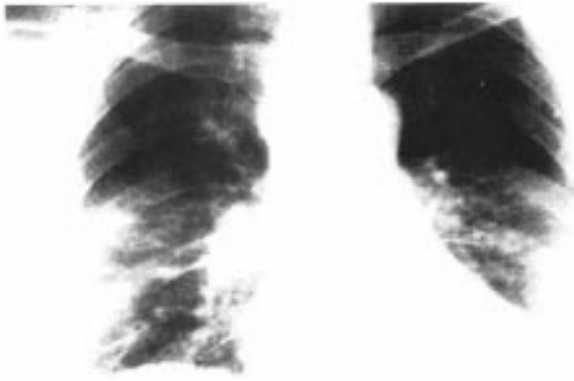
Figure 7. Chest radiogram of patient K.AL. Chronic phase of EAA. Diffuse interstitial changes are seen in the lower and middle lung fields.

Figure 8. Chest radiogram of patient S.S. Acute phase of EAA. Diffuse, confluent interstitial changes are seen in the lower lung fields.

greater than in controls (p < 0.001). The mean percentages of lymphocytes and macrophages in BAL fluid of EAA patients were equal (49.7%) while in controls macrophages were over 10 times more abundant than lymphocytes (p < 0.001). The absolute number of macrophages in BAL fluid was greater in patients than in controls, but the difference was not significant (p > 0.05). By contrast, the numbers of eosinophils and neutrophils were significantly higher in EAA patients (p < 0.001 and p < 0.01, respectively).

DISCUSSION

The results of the present work show that grain dust, in particular from barley, containing large amounts of non-branching bacteria is the commonest offending agent causing allergic alveolitis among agricultural workers in eastern Poland. This is consistent with views indicating the very important role of grain dust in causing respiratory diseases in agricultural populations [27, 33].

Table 4. Results of the analysis of BAL fluid from EAA patients and control subjects.

No.	Initials	Vitality (%)	Yield (%)	Total cells $\times 10^6$	Total cells $\times 10^3$/ml	Macrophages $\times 10^3$/ml (%)	Lymphocytes $\times 10^3$/ml (%)	Eosinophils $\times 10^3$/ml (%)	Neutrophils $\times 10^3$/ml (%)
1.	W.ZB.	88.0	53.3	46.0	575.0	316.2 (55.0%)	257.0 (44.7%)	0.6 (0.1%)	1.2 (0.2%)
3.	R.K.	94.0	53.3	42.7	534.0	373.8 (70.0%)	157.5 (29.5%)	1.6 (0.3%)	1.1 (0.2%)
4.	W.J.	90.0	53.3	44.0	550.0	396.0 (72.0%)	152.8 (27.8%)	0.6 (0.1%)	0.6 (0.1%)
7.	B.J.	91.0	60.0	41.1	457.0	174.6 (38.2%)	280.6 (61.4%)	0.9 (0.2%)	0.9 (0.2%)
8.	W.ZD.	93.0	53.3	46.8	585.0	298.4 (51.0%)	283.1 (48.4%)	1.2 (0.2%)	2.3 (0.4%)
9.	S.Z.	94.0	52.0	46.8	600.0	315.0 (52.5%)	280.2 (46.7%)	0.6 (0.1%)	4.2 (0.7%)
13.	K.AL.	87.0	50.0	43.5	580.0	314.9 (54.3%)	261.0 (45.0%)	1.2 (0.2%)	2.9 (0.5%)
14.	Ł.E.	87.0	66.7	22.0	220.0	92.6 (42.1%)	125.4 (57.0%)	0.7 (0.3%)	1.3 (0.6%)
15.	M.A.	96.0	33.3	10.9	218.0	71.9 (33.0%)	143.9 (66.0%)	0.4 (0.2%)	1.8 (0.8%)
16.	K.K.	89.0	53.3	50.5	631.0	233.5 (37.0%)	391.2 (62.0%)	1.9 (0.3%)	4.4 (0.7%)
17.	K.AN.	91.0	46.7	49.0	700.0	294.0 (42.0%)	399.7 (57.1%)	0.7 (0.1%)	5.6 (0.8%)
20.	K.S.	89.0	43.5	77.4	1191.0	582.4 (48.9%)	597.9 (50.2%)	1.2 (0.1%)	9.5 (0.8%)
Total patients $\bar{x} \pm$ S.D. n = 12		90.7 ± 2.8	51.6 ± 7.8	43.4 ± 15.2**	570.1 ± 236.0**	288.6 ± 131.9 (49.7 ± 11.8%)**	277.5 ± 129.8** (49.7 ± 11.6%)**	1.0 ± 0.4** (0.2 ± 0.1%)	3.0 ± 2.5* (0.5 ± 0.3%)
Total controls $\bar{x} \pm$ S.D. n = 17		91.3 ± 1.9	55.1 ± 6.6	18.0 ± 9.6	220.8 ± 124.0	202.6 ± 114.7 (91.5 ± 2.5%)	17.2 ± 10.1 (8.0 ± 2.5%)	0.4 ± 0.2 (0.2 ± 0.1%)	0.6 ± 0.4 (0.3 ± 0.3%)

* ̄ **: Difference between groups of patients and controls statistically significant. * p < 0.01, ** p < 0.001.

A Gram-negative bacterium *Pantoea agglomerans* and a corynebacterium *Arthrobacter globiformis* were proved as the commonest agents causing disease. Other identified agents were *Alcaligenes faecalis, Brevibacterium linens* and *Staphylococcus epidermidis*.

To date, several species of non-branching bacteria have been identified as causes of allergic alveolitis: *Bacillus subtilis* [19, 31], *Pantoea agglomerans* [10, 23, 35], and *Cytophaga allerginae* [30]. *Alcaligenes faecalis* has also been identified as a possible cause of pulmonary disorders among individuals occupationally exposed to the inhalation of large amounts of herb dust [24]. Nevertheless, non-branching bacteria are generally underestimated as a potential cause of allergic alveolitis among agricultural workers, compared to actinomycetes and fungi. There is a simple reason for this: extracts of extraneous bacteria are not included in the popular antigen panels for diagnostics of allergic alveolitis, and the patients are tested with antigens of *Saccharopolyspora rectivirgula* and other actinomycetes and fungi recognized as a common cause of the disease [14, 17, 41]. So far, *Pantoea agglomerans* and other Gram-negative bacteria are recognized as a source of respirable endotoxin causing Organic Dust Toxic Syndrome (toxic pneumonitis) and acute form of byssinosis [7, 44, 54, 55]. Our results suggest that both Gram-negative and Gram-positive bacteria may also be potential sources of specific antigens causing allergic alveolitis indicating that further studies on this problem are needed.

The results of this work confirm the view of Terho and Lacey [59] on the importance of the microbiological examination of offending dust for proper identification of etiological factors causing allergic alveolitis. They suggest also that the spectrum of these factors may vary from country to country, depending mainly upon climatic conditions. The patients we examined lived in the Lublin Upland region of eastern Poland, characterised by a moderately dry climate. As heavy rainfalls in this region are uncommon, none of the patients reported dust from moist, overheated hay or grain as an offending material causing disease. By contrast, in the areas characterised by a humid climate (British Isles, Scandinavia, the Alpine area) the dust from moist forage containing large amounts of thermophilic actinomycetes and/or fungi is often reported as a cause of farmer's lung and other forms of allergic alveolitis [6, 28].

To the best of our knowledge, Gram-positive bacteria belonging to corynebacteria and staphylococci have not been reported so far as causative agents of allergic alveolitis. Of three species from these groups, identified by us as factors of disease (*Arthrobacter globiformis, Brevibacterium linens, Staphylococcus epidermidis*), *A. globiformis* seems to be the most significant. It clearly dominated in two samples of barley and barley dust (Fig. 1) and was identified as a cause of allergic alveolitis in eight grain elevator workers exposed to barley.

We suggest that much more attention should be paid to *Arthrobacter* spp. as a potential cause of work-related
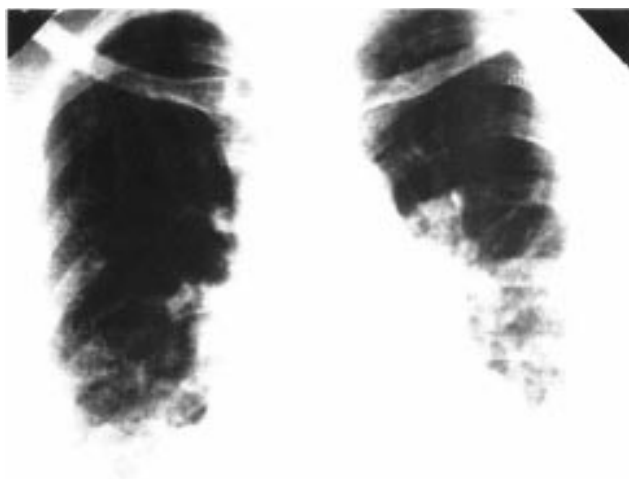

Figure 9. Chest radiogram of patient K.D. Chronic phase of EAA. Interstitial fibrotic changes are seen in the lower and middle lung fields.

respiratory disease in agricultural population. The organism is common not only in grain, but also in straw, different fodders and in the air of animal farms [13, 57]. The *Arthrobacter* organisms are coccoid and rod-shaped in appearance, and distinguish themselves by an unusual process of cell divisions combining binary fission and multiple segmentation (Fig. 11–12) [57]. We have observed that during the cell divisions a fibrillar extracellular substance is released (Fig. 12) which probably contains the offending antigen. This is a different process compared to Gram-negative bacteria, which release offending endotoxin and antigens in the form of membrane vesicles measuring 30–50 nm [12]. The characteristics of the nature and chemical composition of
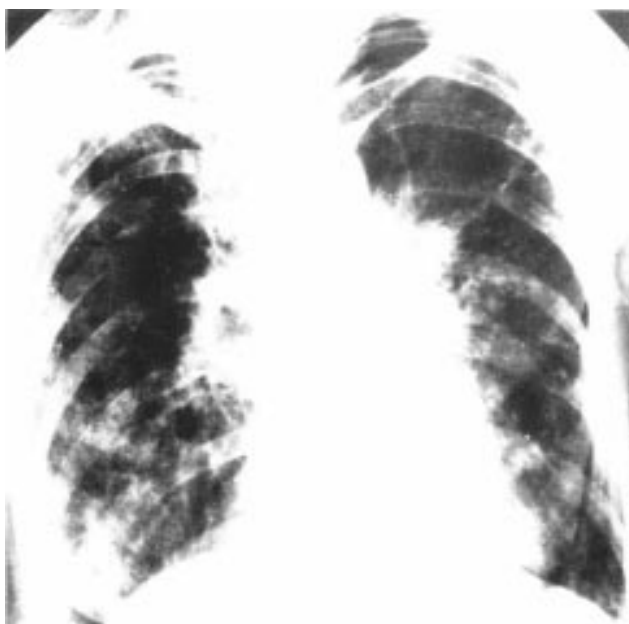

Figure 10. Chest radiogram of patient R.W. Chronic phase of EAA. Fibrotic and emphysematous changes in lung.

this potentially pathogenic substance released by *Arthrobacter* spp. and other Gram-positive bacteria will be the subject of further studies by our group.

The establishing of the ethiological diagnosis of EAA is difficult in many cases and necessitates performing the inhalation challenge [20, 42, 43, 56]. This may be carried out by recreating the work conditions or by exposing a patient to graded concentrations of aerosolised antigen [42]. Patients respond adversely to the offending antigen and experience fever and dyspnea 4–6 hours after exposure. Together with systemic symptoms, there is usually a decrease in the vital capacity and diffusing capacity, and leukocytosis [42, 43]. Müller-Wening *et al.* [43] observed moderate leukocytosis and slight rise of temperature also in healthy subjects exposed to inhalation of hay dust. These changes however, were not accompanied by a decrease of spirometric values.

Positive result of the challenge is very important for diagnosis [56]. In order to perform this test correctly it is essential to select the appropriate antigens and to prepare a proper dilutions before introducing them into the shock organ (lungs). On the basis of this study, it could be stated that inhalation challenge is a valuable and safe test in making diagnosis of EAA.

The results indicate the importance of using a broad spectrum of tests and antigens, including those selected on the basis of environmental studies, for a proper identification of the cause of EAA in individual cases.

The clinical presentation of EAA may be acute or chronic, depending on frequency, intensity and duration of exposure. Both respiratory and systemic symptoms may occur. In the acute form, influenza-like symptoms often predominate, consisting of chills, fever, sweating, myalgias and headache that begin 4-8 hours after exposure and last from hours to days. The chronic form has an insidious onset over a period of months, with increasing cough and dyspnea. Fatigue and weight loss may be prominent symptoms. Our study is based on the clinical observations and immunological tests in 20 cases of EAA. The clinical findings in general confirm the observations of previous investigators [15, 22, 38, 40]. The clinical outcome was relatively moderate and no severe or fatal evolution of the disease was observed. The diagnosis of chronic form of EAA was stated in most
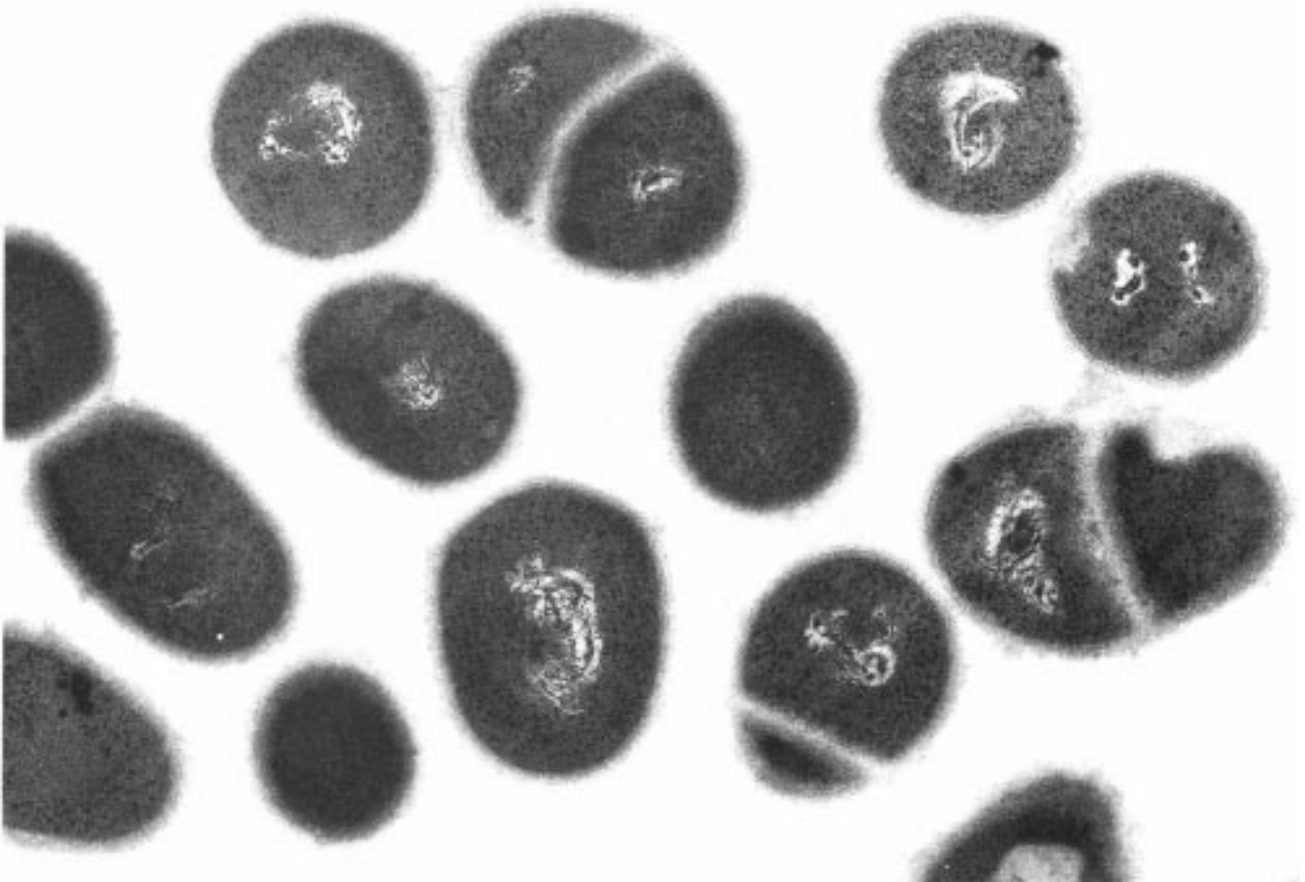

Figure 11. Ultrathin section of the cells of *Arthrobacter globiformis* grown for 48 hrs at 34°C. EM, × 30 000.

patients. During the chronic outcome the acute exacerbations of the symptoms were observed after a heavy exposure to organic dust. These exacerbations were considered as "influenza like" or "common cold" symptoms. According to the opinion of Molina *et al.* [40] and other authors, such a clinical outcome is typical for EAA and may cause some difficulties in proper diagnosis of the disease. The most frequent symptoms in our patients were dry cough and shortness of breath which appeared 4-8 hours after exposure - the typical time-relationship in EAA.

The chest X-ray of EAA patients sometimes appears normal, even in symptomatic patients. The acute phase may be associated with a small, poorly defined, uniform, rather discrete and diffuse nodulation present in both lung fields. Such changes were found in six patients, and in four of them very intensive interstitial infiltrations in all lung fields were found.

In the chronic fibrotic phase, there are radiographic changes of diffuse interstitial fibrosis and honeycombing, sometimes with presence of emphysema. In our group of patients, diffuse interstitial fibrosis was found in four cases. It is noteworthy that haemoptysis occurred in all these patients.

The technique of bronchoalveolar lavage (BAL) has been used to recover cellular and soluble materials from the lung of patients with EAA and there is increasing interest in the significance of this procedure in the evaluation and clinical management of patients with other interstitial lung diseases. BAL fluid in EAA typically shows excess numbers of lymphocytes, a normal number of macrophages and increased number of neutrophils in acute phase of the disease [42, 43, 49, 56]. Our EAA patients demonstrated a significant lymphocytic alveolitis, both in terms of percentage and absolute number. Also, the numbers of eosinophils and neutrophils were significantly higher in EAA patients. These data are consistent with the observations of other authors [20, 42, 43, 49]. A major problem with the analysis of BAL fluid is that many of the abnormalities observed in patients with EAA are also found in antigen-exposed patients without symptoms or clinical evidence of lung disease [42, 43]. Müller-Wening *et al.* [43] found that one-hour exposure to the inhalation of hay dust caused a significant increase of lymphocytes in the BAL fluid of healthy subjects. Kirsten *et al.* [20] reported that BAL
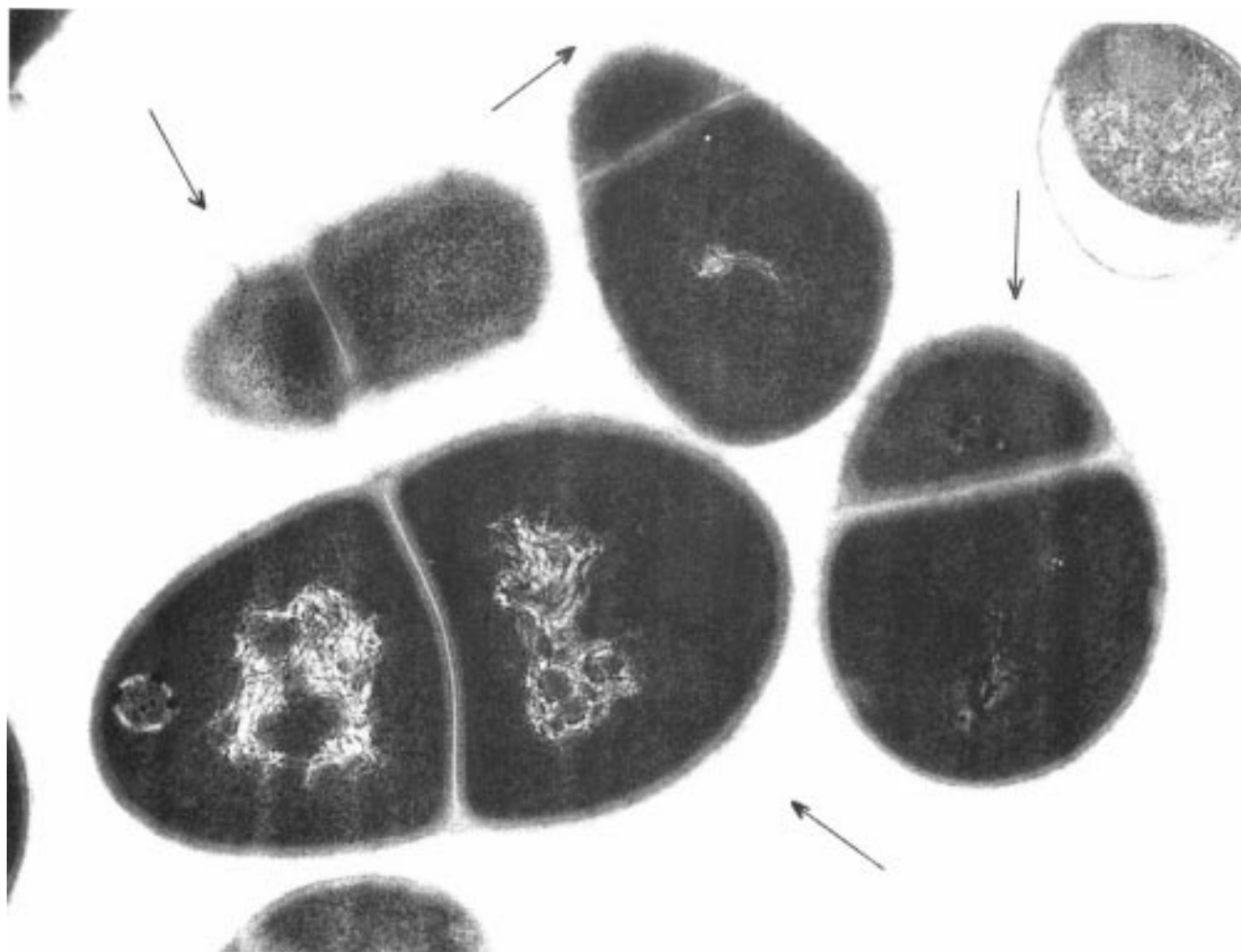

Figure 12. Ultrathin section of the cells of *Arthrobacter globiformis* grown for 48 hrs at 34ºC. Note unusual mode of cell division and a fibrillar substance released from cells (marked with arrows). EM, × 56 000.

lymphocytosis persisted for three years after the remission of clinical symptoms in an EAA patient, despite antigen avoidance. It seems that lymphocytic alveolitis is a common finding in antigen-exposed individuals and the presence of excess lymphocytes alone in BAL fluid cannot be used as a diagnostic marker for EAA.

To summarise, no single test is pathognomonic for EAA - the diagnosis is usually made only after consideration of the occupational history, clinical picture, radiographic evaluation, pulmonary function studies and immunological tests.

CONCLUSION

1. The most common cause of allergic alveolitis among agricultural workers in eastern Poland are mesophilic, non-branching bacteria associated with grain dust, mostly *Pantoea agglomerans* and *Arthrobacter globiformis.*

2. The results indicate the importance of using of a broad spectrum of antigens, including those selected on the basis of environmental studies, and of a broad spectrum of tests including inhalation challenge and BAL, for a proper identification of EAA and its cause in individual cases.

Acknowledgement

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