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High prevalence of mollusc shell hypersensitivity pneumonitis in nacre factory workers

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ABSTRACT: Following the discovery of hypersensitivity pneumonitis caused by the inhalation of mollusc shell dust in two workers from a nacre-button factory, the health status of 26 workers employed in sawing mollusc shells was investigated.

The evaluation included the administration of two questionnaires and radiological, functional and immunological assessments of all workers at the outset and 1 year later, when hygienic and therapeutic measures had been taken.

Six workers, in whom specific inhalation challenge test was positive, were diagnosed with mollusc shell hypersensitivity pneumonitis, thus yielding a prevalence of 23%. Evidence of diffuse lung disease and systemic symptoms was found in these patients. Nonspecific bronchial hyperreactivity was also found more frequently in patients with mollusc shell hypersensitivity pneumonitis. Specific immunoglobulin G (IgG) level and specific skin testing failed to differentiate patients with mollusc shell hypersensitivity pneumonitis from other exposed workers; whereas, nonspecific skin testing, which was impaired in the patients, did differentiate. Bronchoalveolar lavage and transbronchial biopsy performed in patients with mollusc shell hypersensitivity pneumonitis were consistent with the disease. Removal from an environment containing mollusc shell dust was followed by regression of clinical, radiological and functional changes. The clinical picture of the 20 workers who did not present mollusc shell hypersensitivity pneumonitis remained unchanged, but functional decline was observed despite improvement in the environmental conditions of the factory.

This report describes the first series of patients with mollusc shell hypersensitivity pneumonitis studied, and underlines the importance of careful follow-up of workers occupationally-exposed to mollusc shell dust.

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The number of workers in the world exposed to mollusc shell dust is unknown, due mainly to a wide, uncontrolled craft sector dedicated to the manufacture of different products, particularly adornments and costume jewellery. Although respiratory diseases caused by the inhalation of dust from mollusc shells have long been recognized [1], only isolated cases of asthma [2] and hypersensitivity pneumonitis (HP) [3] have been described. To our knowledge, no epidemiological investigation of workers exposed to mollusc shell dust has so far been reported.

We have previously reported two cases of HP caused by the inhalation of dust in a factory where nacre-buttons were manufactured from sea-snail shells [4]. Following the identification of these two cases, a cross-sectional study was undertaken. Hygienic and therapeutic measures were established, and a new evaluation was made 1 year later.

The present study describes the features of mollusc shell hypersensitivity pneumonitis (MSHP). Other possible occupational disorders found in relation to this hazard are also discussed.

Methods

Following the discovery, in 1988, of MSHP in two subjects [4], the workers and management agreed to an investigation of the environmental conditions in the factory, as well as an evaluation of the health status of the workforce.

Environmental assessment

The illness occurred in a building where sea-snail shells of the species *Tectus niloticus* were used as raw material. The activity of the workers consisted of sawing and cutting shells into disks that were later processed into buttons (fig. 1). Each employee's workstation was equipped with a source of chlorinated water, which trickled onto the sawing-point. The water was renewed monthly and flowed round a closed-circuit stemming from a tank. The building did not contain any forced general ventilation system but each workstation was fitted with a cabin with a localized aspiration system. Supplies and

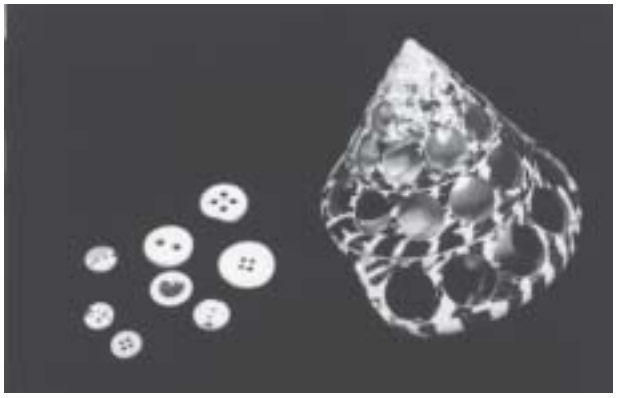

Fig. 1. – Mollusc shell of the species *Tectus niloticus* used to make nacre buttons.

remains of wet shells were stored near each employee for several days. The floor of the building was covered by a fine layer of dust from the workstations and the handling of dry shells.

The presence of endotoxins in the tank water was analysed with the limulus amoebocyte lysate method [5]. One air sample per worker was collected in 1989 to determine the respirable airborne dust level, and was analysed according to standard methods established by the National Institute for Occupational Safety and Health USA [6]. These samples were collected by a Fixt-flow (Mine Safety Appliances, Pittsburgh, USA) personal sampling pump, with a flow of 2 $L \cdot min^{-1}$. The total sample volume of 100 L was representative of the work cycles of each worker during the working day. Dust concentration was determined by the gravimetric technique. Dust from one sample was analysed by X-ray diffraction. Cross-reactivity of allergens in extracts from another two samplers and the sea-snail extract were studied by radioimmunoassay (RIA) inhibition analysis [7].

Cross-sectional study

In addition to a medical history, each worker was requested by a trained interviewer to complete two questionnaires: a modified version of the standard American Thoracic Society (ATS)-DLD-1978 respiratory questionnaire, including a smoking questionnaire [8], and a questionnaire to assess HP, adapted from the criteria of GRANT *et al*. [9] for the study of farmer's lung.

Physical examination, chest radiography and pulmonary function tests were also performed, and a venous blood sample was taken. Spirometry was performed with a Datospyr spirometer (Siebel SA, Barcelona, Spain), according to the instructions of the American Thoracic Society [10] both for the spirometric test and calibration. Predicted normal values were obtained according to ROCA *et al*. [11].

The nonspecific bronchial provocation test was derived from that described by CHATHMAN *et al*. [12]. Briefly, the test was performed, with the subject seated, using a De Vilbiss 646 nebulizer (Health Care UK Ltd, Heston, UK) at a flow rate of 6 $L \cdot min^{-1}$. The subjects were instructed to take slow vital capacity inhalations from functional residual capacity (FRC). Bronchial challenge was performed provided that a patient's baseline forced expiratory volume in one second (FEV_1) was at least 60% of the predicted value. Inhalation of methacholine diluent was followed by successive doses of methacholine, consisting of one breath of 5 $mg \cdot mL^{-1}$, followed by four additional breaths of 5 $mg \cdot mL^{-1}$ of methacholine, then one breath of 25 $mg \cdot mL^{-1}$, and finally four breaths of 25 $mg \cdot mL^{-1}$. Forced vital capacity (FVC), FEV_1 and forced expiratory flow during the middle half of forced vital capacity ($FEF_{25–75}$) were measured by the spirometer before the challenge, and 3 min after inhalation of methacholine diluent and each dose of methacholine. The best of three attempts was recorded, according to the value of the sum of FVC plus FEV_1. Bronchial challenge was considered positive when FEV_1 fell by at least 20%, or $FEF_{25–75}$ by 25% (if FVC did not decrease) from the initial value.

An antigenic extract of sea-snail shells was prepared as follows: sea-snail shells were ground in a mortar until a powder was obtained. This was diluted to 10% in ammonium bicarbonate buffer, 0.2 M, pH 7.9, and shaken overnight. After centrifugation and dialysis against distilled water, with a cut-off 3,500 MW membrane (Spectrapor 3 MNCO; Iberlabo, Los Angeles, CA, USA), the resulting solution was ultracentrifuged for 15 min at 600 ×g until a clear extract was obtained. The protein concentration, determined by the BCA method (BCA Protein Assay Reagent, Pierce, Rockford, IL, USA), ranged 700–1,000 $\mu g \cdot mL^{-1}$.

Immunoglobulin G (IgG) antibodies against this extract were measured by means of a modified enzyme-linked immunosorbent assay [13]. In brief, high-binding microtitre plates were incubated with 2 µg $extract \cdot well^{-1}$ in 0.2 M $Na_2CO_3/NaHCO_3$, pH 9.6, at 4°C, overnight. The plates were then washed (0.1 M phosphate-buffered saline (PBS), pH 7.5/0.005% Tween 20) and blocked (PBS/1% bovine serum albumin (BSA)). After washing, the plates were incubated in duplicate with sera at an appropriate dilution for 2 h at 37°C. After washing, horseradish peroxidase (HRP)-labelled anti-human IgG (clone MH16-1ME, 1 $\mu g \cdot mL^{-1}$) was incubated for 2 h at 37°C. Following washing, the reaction was developed with 3,3',5,5'-tetramethylbenzidine, 3% H_2O_2, for 20 min at room temperature. The reaction was stopped with 2 M H_2SO_4 and read at 450 nm with a plate reader (Titertek Multiskan Plus MKII; Flow Laboratories Ltd, Herts, UK). Results were expressed as units of optical density at 450 nm. Within-run and day-to-day coefficients of variation were 9.4 and 11.6%, respectively. For the *in vivo* test, the antigenic extract was diluted in saline at a protein concentration of 0.01 $mg \cdot mL^{-1}$ and sterilized by filtration with a 0.22 µm pore size filter (Millipore SBGS25SB; Millipore Iberica, Molsheim, France).

Skin tests were performed by means of intradermal injection of 0.1 mL of each antigenic extract [14, 15]. Specific skin test was performed by injection of shell extract (1:100 dilution; 7–10 $\mu g \cdot mL^{-1}$). The test was read after 15 min (immediate reaction), after 4–6 hours (late reaction), and after 48 h (delayed reaction). The test was considered positive when the wheal was greater than 10 mm in the immediate reaction, and when induration was greater than 10 mm in the late and delayed reactions. Nonspecific skin tests were carried out with five antigenic extracts: candidine (Leti Laboratories), 1:100; staphylococcal toxoid (Leti Laboratories), 1:100;

tuberculin purified protein derivative (PPD) (Materiales y Reactivos Laboratories), 5 U tuberculin·0.01 mL^{-1}; trichophyton (Leti Laboratories), 1:100; and streptokinase-streptodornase (Lederle Laboratories), 4 and 1 U·0.1 mL^{-1}; respectively. An individual was considered to be a reactor to nonspecific skin tests when presenting at least one induration of ≥5 mm to any of the five nonspecific antigens tested. Specific IgG antibodies and skin tests were also assayed in 30 healthy volunteer controls, with no apparent previous respiratory exposure to the antigen.

Eleven workers reporting a relationship between the presence of any symptom and the work schedule were recalled for specific inhalation challenge testing. The test consisted of the inhalation of 2 mL of nebulized antigen extract at 1:100 dilution (7–10 µg·mL^{-1}) through a Bird Mark-8 respirator (Palm Springs, CA, USA). The extract was completely inhaled with approximately 15 min of tidal breathing. A control challenge test with the same amount of diluent was also performed on the previous day. The test was considered positive if there was a delayed decrease of over 15% in FVC and/or a decrease in transfer factor of the lungs for carbon monoxide ($T_{L,CO}$) of over 20% compared with prechallenge values. Spontaneous patient-reported symptoms, chest examination and axillary temperature were recorded at hourly intervals. Chest radiography was performed and a blood sample taken when the test proved positive, or at the end of the test if negative. Challenge testing was repeated at 1:10 (70–100 µg·mL^{-1}) and 1:5 (140–200 µg/mL^{-1}) in the event of a negative result.

Six workers with a positive specific inhalation challenge test were considered MSHP patients and underwent bronchoalveolar lavage (BAL) and transbronchial biopsy 2 weeks later. BAL was performed with a BF-B3R Olympus bronchoscope Olympus, Tokyo, Japan. The bronchoscope was wedged in a segment of the right lobe and a total of 150 mL of sterile 0.9% saline solution was injected in 50 mL aliquots, with immediate vacuum aspiration after each aliquot. Cells recovered from the two last aliquots were counted in a haemacytometer. A differential count (made from total counts of 200 cells) was accomplished by morphological criteria in cytocentrifuged smears stained with May-Grünwald Giemsa. Cells were washed with Hank's solution (Gibco, Paisley, UK) and resuspended in PBS with 0.1% sodium acid at 3–10×10^4 cells·$test^{-1}$. Subsets anti-CD3, CD4 and CD8 (Becto Dickinson, Madrid, Spain) were used. After 20 min at room temperature in darkroom incubation, stained lymphocytes were analysed in a fluorescence-activated cell sorter (FACS) system (FACSTAR plus; Becton Dickinson). Results are expressed as percentage of total lymphocytes present in the sample. Transbronchial biopsy specimens were fixed in formalin, embedded in paraffin, and stained with haematoxylin and eosin. Fibrosis was studied using trichromic stain.

Intervention and follow-up

As a result of the study, workers diagnosed with MSHP were released from work, and the following environmental measures were recommended: 1) check the air-extraction system pipes; 2) clean, regularly renew, and disinfect the water supply system with bleach; 3) maintain mollusc shells moist in storage areas; 4) remove the dust from the floor and work surfaces with daily dampening; and 5) provide workers with personal airway protection equipment.

The factory was subsequently visited on four occasions to check and reinforce application of the measures recommended. One year after intervention, the cross-sectional study, including personal interviews, physical examination, chest radiography, spirometry and specific IgG antibodies, was repeated. Air samples were also collected and analysed as described previously.

Data analysis

Data are presented as means (SD) and percentages of positive results. Student's two-tailed test and the Mann-Whitney U-test were used to assess the significance of differences in the quantitative data. Chi-squared and Fisher's exact tests, when required, were used for the qualitative data. A p-value less than 0.05 was considered significant.

Results

Environmental assessment

MSHP subjects were exposed to a nonsignificant lower mean (SD) respirable airborne dust level of 0.93 (0.58) mg·m^{-3} in comparison with that of the non-MSHP workers, which was 1.56 (1.26) mg·m^{-3}. Calcium carbonate, such as aragonite and calcite, was found in the sample analysed by X-ray diffraction. Although different allergen concentrations were found in the two sampler extracts in cross-inhibition studies, the slopes of the inhibition curves were similar, showing comparable allergen components in the two samplers and in the sea-snail shell extract (fig. 2). Endotoxins were found in the tank water.

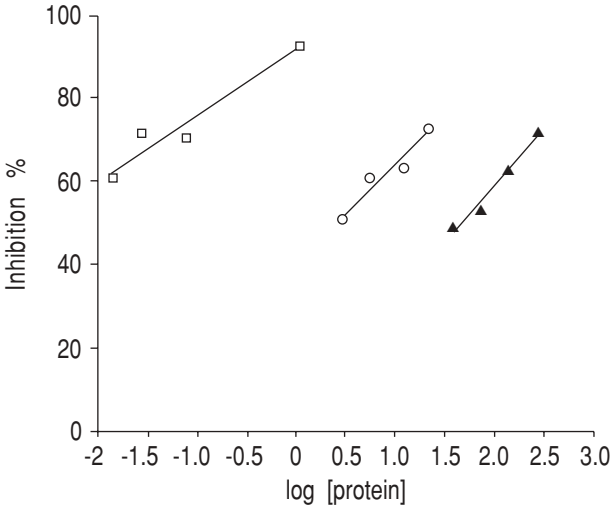

Fig. 2. – Cross-inhibition studies for specific immunoglobulin G (IgG) using sea-snail shell extract as the solid phase and two personal sampler extracts (▲ and ○) and sea-snail extract (□) as fluid phases.

Table 1. – Characteristics of mollusc shell hypersensitivity pneumonitis (MSHP) patients and other workers in the nacre-button factory

Characteristics	MSHP patients (n=6)	Other workers (n=20)	p-value
Sex M/F	1/5	15/5	<0.05
Age yrs	42 (11)	40 (12)	NS
Smokers n	0	9	NS
Exposure duration yrs	16 (2.1)	13.9 (4.3)	NS
Cough n	6	13	NS
Expectoration n	5	10	NS
Chronic bronchitis n	5	9	NS
Reported wheezing n	5	7	NS
Dyspnoea n	4	3	<0.05
Myalgia n	6	1	<0.001
Weight loss n	4	0	<0.0001
Fever n	6	1	<0.001
Rales n	5	0	<0.001
Wheezing on auscultation n	2	2	NS
ESR $mm \cdot h^{-1}$	35.7 (25.1)	7.1 (2.6)	<0.05
Gammaglobulin level^{+} $g \cdot dL^{-1}$	1.47 (0.47)	1.18 (1.34)	NS
Total IgE^{++} $KU \cdot L^{-1}$	46.7 (43.7)	56.6 (69)	NS
Specific IgG$^{\#}$	1.420 (0.026)	0.941 (0.608)	NS
Diffuse pattern radiograph n	4	0	0.001
FVC % pred	80.7 (14.9)	91.6 (12.2)	NS
FEV_1 % pred	80.5 (13.4)	90.7 (14)	NS
FEV_1/FVC %	75.2 (7)	77.2 (8.5)	NS
Positive methacholine test	5/6	4/17	<0.05

Values are presented as absolute number, or as mean and SD in parenthesis. $^{+}$: normal value 0.7–1.5 $g \cdot dL^{-1}$; $^{++}$: reference value 10–120 $KU \cdot L^{-1}$; $^{\#}$: optical density at 450 nm (reference value 0–0.314 units of optical density). M: male; F: female; ESR: erythrocyte sedimentation rate; IgE and IgG: immunoglobulins E and G; FVC: forced vital capacity; FEV_1: forced expiratory volume in one second; % pred: percentage of predicted value [11]; NS: nonsignificant.

Cross-sectional study

The nacre-button factory employed 26 workers, including the two index cases (table 1).

Six workers were diagnosed with MSHP, which represents a prevalence of 23%. Their mean (SD) duration of symptoms prior to diagnosis was 4.7 (0.8) (range 0.5–6) yrs. All patients reported that respiratory and systemic symptoms appeared or worsened in the second half of working days. Chest radiographs showed a diffuse alveolar filling lesion in one patient, and an interstitial diffuse pattern in another three. Three of four MSHP patients, for whom spirometry was available during the time they were actively working, presented decreased FVC. Spirometric data of the two index cases had not been collected at that time.

A higher proportion of subjects with a significant specific IgG level against the shell extract was observed among the MSHP patients compared with nonexposed control subjects; however, no significant difference was observed between MSHP patients and the remaining workers. Specific skin tests failed to differentiate MSHP patients from remaining workers or control subjects, but MSHP patients exhibited much lower nonspecific cutaneous hyperreactivity (table 2).

Inhalation challenge tests with the antigen extract were positive in 6 of the 11 workers, who had been tested because they had reported work-related symptoms. Five reactions were of the late type, and one was dual (fig. 3). The mean maximum fall in FVC of 21% after positive tests was accompanied by a similar mean maximum fall in FEV_1 of 23%, suggesting a restrictive ventilatory disorder. Mean FEV_1/FVC ratios were similar both immediately before challenge and at the time of the maximum fall in FVC. Appreciable decreases in $T_{L,CO}$ (>20%) occurred in these six workers. All six also developed symptoms similar to those they reported whilst working in the factory, accompanied by fever in five cases. Moreover, a fall in lymphocyte count of 500 $cells \cdot mm^{-3}$ or more, coupled with the development of absolute lymphopenia (<1,500 $lymphocytes \cdot mm^{-3}$) was recorded in three patients.

Table 2. – Specific immunoglobulin G (IgG) antibodies and skin reactivity in mollusc shell hypersensitivity pneumonitis patients (MSHP), other exposed workers and nonexposed healthy control subjects

	MSHP patients (n=6)	Other workers (n=20)	Healthy controls (n=30)
Specific IgG^{+}	6 (100)†	15 (75)	2 (7)
Skin reactivity^{++}			
Specific immediate	5 (83)	8 (50)	10 (33)
Specific late	2 (33)	0 (0)	3 (10)
Specific delayed	0 (0)	0 (0)	0 (0)
Nonspecific delayed	1 (17)†‡	16 (100)	30 (100)

†: p<0.001 *versus* healthy control subjects; ‡: p<0.001 *versus* other workers; $^{+}$: number of individuals with a significant (mean control value +2SD) level; $^{++}$: number of positive reactions. Four non-MSHP workers refused to undergo the skin tests. Values are presented as absolute number, and percentage in parenthesis. NS: nonsignificant.

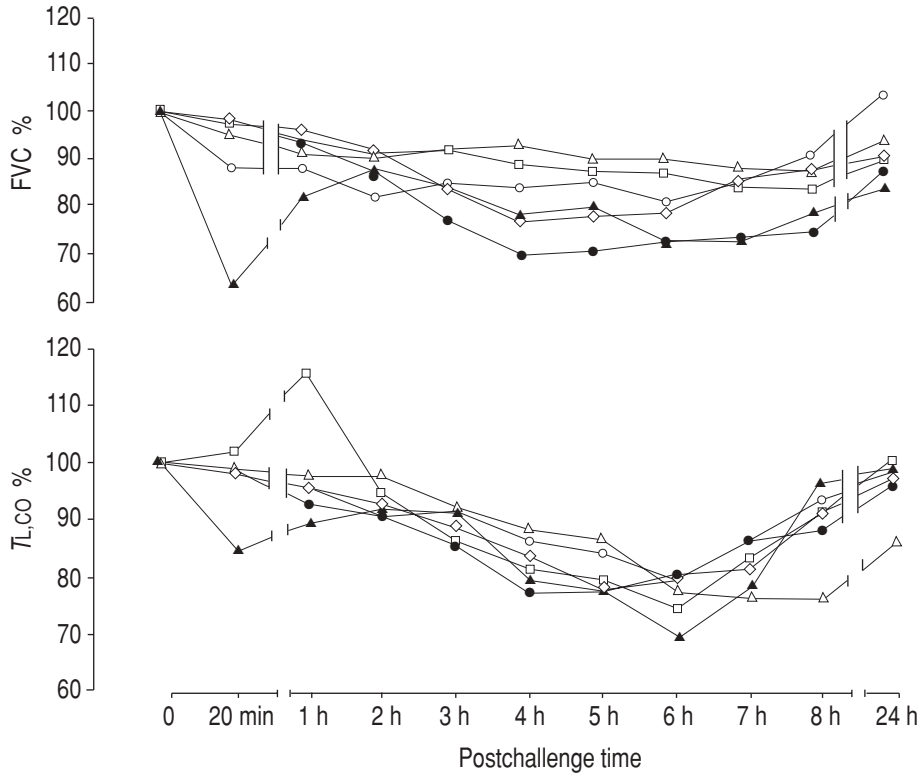

Fig. 3. – Positive inhalation challenge tests. Forced vital capacity (FVC) and transfer factor of the lungs for carbon monoxide ($T_{L,CO}$) after challenge, as a percentage of baseline values. Five reactions were of a late type, and one of a dual type (▲).

The mean absolute number of cells recovered from BAL was 62.5 (5.7) $\times 10^6$ cells·100 mL^{-1}, with the following mean differential count: 36 (13)% macrophages; 58 (16)% lymphocytes; 5 (3)% neutrophils; and 1 (3)% eosinophils. The mean percentage of lymphocytes staining for: CD3 was 79 (14)% for CD4 23 (13)%; and for CD8 43 (7)%. The mean ratio of CD4+ to CD8+ T-cells was 0.5 (0.1). Transbronchial lung biopsy showed an interstitial alveolar infiltrate of plasma cells and lymphocytes, sometimes in clusters, predominantly at the centre of the acinus in the six patients. Interstitial fibrosis was also observed in two patients, and granulomata in another two. Amyloid deposits were observed in one patient described previously [16].

Among the 20 workers who did not present MSHP, 14 (70%) reported symptoms, although eight of them were nonsmokers. None had a history of acute, reversible attacks of dyspnoea. These symptoms were activity-related in five workers, but none had significant chest radiograph, or pulmonary function alterations, or positive specific inhalation challenge test. No symptoms, fever, significant changes in chest examination or chest radiograph, or in leucocyte count were recorded postchallenge in these five workers.

Follow-up

One year after removal from work, all MSHP had become asymptomatic and, with the exception of specific IgG which remained at a similar level (1.410± 1.003 units of optical density), other tests performed were normal.

Non-MSHP workers continued to work in the factory. Environmental measures recommended in the factory were correctly applied, although personal airway protection was used inconsistently. One year later, a decrease (p=0.06) in airborne dust level (0.91 (1.95) mg·m^{-3}) was verified. One worker who had previously referred to cough no longer reported this symptom. Symptomatic changes were not found in the remaining workers. No radiological or significant changes in specific IgG level (0.966±0.613 units of optical density) were observed. After one year, non-MSHP workers had significant drops in FEV_1 and FVC (table 3).

Discussion

The main aim of this study was to describe the features of MSHP. We, therefore, selected the case definition that was believed to be highly specific, in order to minimize the likelihood of including false positive cases. Subjective and objective changes observed in these patients after the inhalation test have proved to be highly specific for the diagnosis of HP [17]. Furthermore, the existence of a high specific IgG level, consistent BAL and histological features, and improvement in the disease following removal from the antigenic source, support the diagnosis of HP. However, since specific bronchial challenge test was performed only in workers with work-related symptoms, and false negatives were possible among the tested subjects, the existence of MSHP in other workers cannot be entirely ruled out. Nevertheless, the 23% prevalence encountered appears to be high

Table 3. – Spirometric data of mollusc shell hypersensitivity patients (MSHP) and other workers at entry to the study and after intervention

Spirometric data	MSHP		Other workers	
	Entry	1 year	Entry	1 year
FVC % pred	81±15 (59–93)	98±10 (84–107)*	92±12 (67–112)	87±12 (60–107)***
FEV_1 % pred	80±13 (62–92)	94±15 (79–114)	91±14 (65–109)	86±13 (56–108)***

FVC: forced vital capacity; FEV_1: forced expiratory volume in one second; % pred: percentage predicted [11]. Values are mean±SD and range in parenthesis. *: p<0.05; ***: p<0.001, compared to value at entry to study.

compared with the 0.5–15% in other studies of HP [18], although prevalence rates as high as 70% have been reported in HP outbreaks [19]. The conditions determining these wide variations are not well-known. Some reports [20, 21] have suggested that some inflammatory event must occur in the lung, in addition to recurrent antigen exposure, for HP to develop. Microbial contamination found in the tank water was ruled out as an antigenic cause of MSHP in the two index cases [22]. However, microbial toxins [23] and other products in the factory could have facilitated the occurrence of mollusc shell HP, due to an irritative lung inflammatory effect.

Taking into account that all of the MSHP patients were nonsmokers, smoking seems to have had a protective effect against the appearance of this disease, as has been mentioned in other HP [24]. Clinical presentation of diffuse lung disease and systemic symptoms differentiated MSHP patients from other workers in the factory. Nonspecific bronchial hyperreactivity, and nonsignificant chronic bronchitis and wheezing were found more frequently in MSHP patients than in the other workers. In addition to the antigen, calcium carbonate [25], the inorganic constituent of mollusc shells, and the presence of microbial toxins [26], might also have contributed to the bronchial involvement found in this nacre factory.

Irritant effects in the immediate response and the lack of positive results in late and delayed responses render specific skin tests useless in the diagnosis of MSHP. In contrast, nonspecific delayed cutaneous hypersensitivity was impaired in MSHP patients, but not in the other exposed workers or control subjects. Patients with HP have a decreased CD4/CD8 cell ratio in peripheral blood due to a decrease in the number of CD4+ cells and an increase in CD8+ cells [27]. However, non-HP-exposed workers have a normal CD4/CD8 cell ratio, with normal CD8+ cell number but slightly decreased CD4+ cells [28]. This supports the idea that suppressor cell activity might differ in symptomatic workers compared with those who are asymptomatic [29], and that this activity could be responsible for the cutaneous anergy associated with the disease as observed in other forms of HP [15].

No evidence of disease was observed in these MSHP patients after one year of nonexposure to mollusc shell dust. Early elimination of the causal antigen appears essential to avoid progression of HP [30]. On the other hand, some studies [31, 32] have shown that workers exposed to inorganic and organic dust have increased annual loss of pulmonary function. Given the loss of respiratory function in the non-HP workers, it is possible that even the improved hygienic conditions did not suffice to change this tendency in some workers. Moreover, interaction between occupational exposure and cigarette smoking might have contributed to the decline of pulmonary function [32]. These facts underline the importance of careful follow-up of workers occupationally-exposed to mollusc shell dust.

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