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Hypersensitivity Reactions and Specific Antibodies in Workers Exposed to Industrial Enzymes at a Biotechnology Plant*

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Thirty-six employees who produced industrial enzymes from selected strains of bacteria and fungi were evaluated by epicutaneous threshold testing and enzyme-linked immunosorbent assays (ELISA) for specific IgE and IgG antibodies. The workers complained of 'asthma- and flu-like' symptoms, which generally lessened away from work. The enzymes evaluated were: α-amylase (1,4-α-$_d$-glucan glucanohydrolase) from *Bacillus* licheniformis (α*ABl*), B. subtilis formation 1 (α*A1Bs*) and B. subtilis formation 2 (α*A2Bs*); purified α-amylase from B. licheniformis (C*αABl*) and A. oryzae (C*αAAo*); alkaline protease from B. licheniformis (AP*Bl*) and purified alkaline protease (CAP*Bl*); amyloglucosidase (1,4-α-$_d$-glucan glucohydrolase) from A. niger (AG*An*) and purified amyloglucosidase (CAG*An*). Statistically significant increases ($P > 0.05$) in the proportion of workers having positive skin tests to CAPBl, AG*An* and CAG*An* were found. Significantly elevated ($P > 0.05$) mean specific IgE results were observed for C*αAAo* CAG*An* and AG*An*, and elevated ($P > 0.05$) mean specific IgGs were observed for C*αAAo*, CAG*An*, AG*An*, α*A1Bs*, αAB1 and αA2Bs. These results indicate that occupational exposure to some industrial enzymes can cause immediate-onset cutaneous hypersensitivity reactions, pulmonary function deficits and significantly elevated specific antibody levels. Our results are equivocal as to whether work-related respiratory and cutaneous hypersensitivity reactions are antibody mediated, as there was no statistically significant association between these reactions and specific IgE or IgG levels.

INTRODUCTION

For many years, a variety of bacterial and fungal microbial products as well as host organisms have been recognized as important etiological agents of occupational allergies and respiratory disorders.[1–11] The sensitizing potential of these types of enzymes is evidenced by the American Conference of Governmental Industrial Hygienists (ACGIH) threshold limit value (TLV) of 0.00006 mg m^{-3} (ceiling value) for 100% pure subtilisins (an enzyme from Bacillus subtilis), which is by far the lowest TLV for a chemical.[18]

Cases of hypersensitivity pneumonitis have also been documented in individuals exposed, in the occupational environment, to fungi, thermophilic actinomycetes and animal proteins.[12–17]

In the present investigation we describe the results of epicutaneous threshold testing, enzyme-linked immunosorbent assays (ELISAs), pulmonary function testing and symptom questionnaires from workers employed at a facility which produced multiple industrial enzymes from numerous strains of bacteria and fungi.

METHODS AND MATERIALS

Process description and exposures

The workers studied are emloyees who worked in the production of industrial/commercial grade enzymes that have been cultivated from selected strains of B. licheniformis, B. subtilis and Aspergillus niger. Enzymes produced at this plant are packaged in liquid formulations of varying strengths that are destined for use in detergents, food processing and textile manufacturing. While the exact process and nutrients used in the porduction of these enzymes is proprietary, the following process description is a generalized overview of the production steps. Pure strains of microorganisms are combined with specially selected sterile nutrient media (selected to provide protein and nitrogen sources) in a 'seed tank' where biological amplification can occur. Once sufficient biomass has accumulated,

* Mention of a product or company name does not constitute endorsement by the National Insitute for Occupational Safety and Health (NIOSH).

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the culture is aseptically transferred to a large fermentation vessel. The mixture of nutrients and microorganism is allowed to undergo fermentation and enzyme production. Enzymes are separated from the biomass through a series of filtration steps. The enzyme slurry is pumpled to the filter system where a major portion of the suspended solids is separated from the enzyme liquid. The solid wastes from these operations are discharged into dumpsters and transported to landfills. The enzyme liquid is concentrated with an evaporator and refiltered to remove unwanted bacterial contamination. Following filtration, enzyme activity is stabilized and preservative materials are added to the product during the last phase of production.[23]

Study population

The present investigation was part of a NIOSH Health Hazards Evaluation (HHE) of workers employed in a factory which produces industrial and food-grade enzymes by fermentation biosynthesis and recovers the products by extraction and purification. This evaluation was conducted in response to employee complaints of irritated eyes, skin and respiratory tract when working with certain enzymes. All workers were invited to participate in the study. Thirty-six workers (72% male), representing 65% of the available work force, participated. Participants had a mean age of 43 years (range: 24–59 years) and worked at the plant for a mean of 17 years (range: 7 months–38 years). These workers had been employed in enzyme production for an average of 5.2 years (range: 9 months–17 years). Participants included: 12 process operators (33% of the study participants), seven laboratory technicians (19%), four maintenance workers (11%), four supervisors/foremen (11%) and two electricians (6%), all with direct exposure to the enzymes, while three secretaries (8%), two shipping and receiving personnel (6%) and two others (6%) had casual exposures.

In addition, control skin prick test results from 14 and sera from three normal volunteer control subjects from the Cincinnati, Ohio area were included. Informed consent was obtained from all study participants and the study protocol was approved by the Institute's Human Subjects Review Board.

Antigens

The enzymes evaluated included five process formulations obtained from the plant and four commercially available (Sigma Chemical Co., St. Louis, MO) purified enzymes (Table 1). The workers were also epicutaneous threshold tested with a panel of five common aeroallergens (atopy screen), including blue grass, cat dander, alternaria and short ragweed (Hollister-Stier, West Haven, CN).

Environmental measurements

Exposures were characterized by collecting air samples for alkaline protease ($N = 38$) and amyloglucosidase ($N = 26$) enzyme activity and measurements of total protein ($N = 24$) (26). Air samples were collected on Teflon filters (37 mm, 1.0 μm pore size) using a flow rate of 3 l min^{-1}. For amyloglucosidase, the samples were analyzed by a method based on principle that amyloglucosidase hydrolyses *p*-nitrophenyl-α-$_d$-glucopyranoside to *p*-nitrophenol and glucose when incubated at 55 °C and a pH of 4.3. The liberated *p*-nitrophenol is measured by adjusting the incubated solution to a pH of 8 and measuring the absorbance 400 nm. The amount of *p*-nitrophenol liberated is directly proportional to the amount of amyloglucosidase present. Units are expressed as the enzyme activity

Table 1. Abbreviations, sources and enzyme commission numbers and common and systematic names of antigens

Abbreviation	Common name	E.C. number and systematic name[a]	Microbial and (acquisition[b]) source
Ap*Bl*	Alkaline protease	E.C. 3.4.24. (microbial metalloproteinase)	B. licheniformis (workplace)
αA*Bl*	α-Amylase	E.C. 3.2.1.1. (1, 4-α-D-glucan glucanohydrolase)	B. licheniformis (workplace)
AG*An*	Amyloglucosidase	E.C. 3.2.1.3. (1, 4-α-D-glucan glucohydrolase)	A. niger (workplace)
αA1*Bs*	α-Amylase	E.C. 3.2.1.1 (1, 4-α-D-glucan glucanohydrolase)	B. subtilis formulation 1[c] (workplace)
αA2*Bs*	α-Amylase	E.C. 3.2.1.1 (1, 4-α-D-glucan glucanohydrolase)	B. subtilis formulation 2[c] (workplace)
CAP*Bl*	Alkaline protease	E.C. 3.4.24 (microbial metalloproteinase)	B. licheniformis (commercial)
CαA*Ao*	α-Amylases	E.C 3.2.1.1 (1, 4-α-D-glucan glucanohydrolase)	A. oryzae (commercial)
CαA*Bl*	α-Amylases	E.C. 3.2.1.1 (1, 4-α-D-glucan glucanohydrolase)	B. licheniformis (commercial)
CAG*An*	Amyloglucosidase	E.C. 3.2.1.3 (1, 4-α-D-glucan glucanohydrolase)	A. niger (commercial)

[a] Enzyme Commission (E. C.) numbers and systematic names were obtained from either the WWW site: http: // expasy. hcuge. ch/sprot/enzyme. html [27] or Sigma Chemical Company, St. Louis, MO.
[b] Acquisition refers to source of the enzyme, either from the workplace or commercially from Sigma Chemical Co., St. Louis, Mo.
[c] Refers to two distinctly different formulations of α-amylase.

which will catalyse the production of 1 g of glucose in 1 h; the limit of quantitation for this assay was 0.48 U. For alkaline protease, the method used was based on the principle that protease cleaves the peptide bonds of an *N,N*-dimethylcasein substrate when incubated at 55 °C and a pH of 8.5. Primary amino groups are produced which are reacted with trinitrobenzenesulfonic acid at 55 °C to produce a yellow color. The intensity of the color change is measured at 450 nm and is proportional to the amount of protease activity present. Units are expressed as the enzyme activity which will liberate four micromoles of tyrosine per minute; the limit of quantitation (LOQ) of this assay was 2.0×10^{-3} U. Total protein samples were collected on 37-mm glass-fiber filters at a flow rate of 3 l min^{-1} and analyzed by a micro Kjeldahl nitrogen technique.[26]

Questionnaire

Past employment information, medical history, use of medications and the presence and frequency of symptoms were all determined through the use of a self-administered questionnaire.[26]

Epicutaneous threshold titration testing

For epicutaneous testing, 100-μl drops of test solutions (diluted in sterile phosphate-buffered saline (PBS: 0.02 M phosphate buffer (pH 7.4) containing 0.9% NaCl) were carefully placed on the volar aspects of both forearms and pricked with a 26-ga hypodermic needle. After a period of 15 min, the areas were observed for wheal and flare responses. Reactions were considered positive when there was a wheal and flare (the wheal being measured using a calibrated caliper, with the wheal having a diameter < 3 mm more than the diluent control test). Atopy was defined by positive results to two or more of the common aeroallergens. Serial tenfold dilutions of the commercial enzymes were used: CAP*Bl*, CαA*Ao*, CαA*Bl* and CAG*An* in PBS, from 10^{-3} to 1 mg ml^{-1}; AP*Bl*, αA*Bl*, AG*An* and αA1*Bs* at 5 mg ml^{-1} and αA2*Bs* at 3.4 mg ml^{-1}. Skin testing was performed with all the antigens by the University of Cincinnati Allergy Research Laboratory. Volunteers with no occupational exposure to enzymes (14 individuals) served as skin testing controls. Final test dilutions were the lowest that failed to elicit a positive response in these control subjects.

Pulmonary function testing

Pre-shift pulmonary function tests were offered to each participant. In addition, process operators and foremen who were exposed to enzymes were selected to receive post-shift pulmonary function tests. Pulmonary function tests were conducted using dry rolling seal spirometers equipped with direct reading terminals and dedicated microprocessors. Pulmonary function testing procedures conformed with the American Thoracic Society's criteria for screening spirometry.[19] One-second forced expiratory volume (FEV_1) and forced vital capacity (FVC) were measured, and the ratio FEV_1/FVC was calculated for each participant. Predicted values were calculated using Knudson's equations and 0.85% was used as a correction factor for evaluating predicted FEV_1 and FVC values for black participants.[20] In addition to the tests previously described, a Mini-Wright portable peak flow device was distributed to each participant following a demonstration of proper use and accurate measurement and recording of results. Each participant was then asked to show an understanding of the proper technique for measuring peak flow by completing one measurement and recording the result prior to leaving the testing area. Additionally, each participant received a sheet of written instructions as a reminder of proper technique. Workers were asked to record the results of three attempts, every 3 h while awake, for 7 days.

Enzyme-linked immunosorbent assay (ELISA)

Blood was obtained by venipuncture and sera stored at −20 °C until the immunoassays were performed. Specific IgG and IgE antibodies to each process and commercial enzyme were measured by modified indirect ELISA methods.[21] Briefly, 5 μg of each enzyme diluted in 0.1 M $NaHCO_3$ (pH 8.6) was placed in each well of a 96-well microtiter plate and incubated at 4 °C overnight. The plates were washed three times with PBS and a similar wash was repeated between all subsequent steps. Aliquots (200 μl) of each diluted serum sample (1:10 in 5% bovine serum albumin (BSA)–deionized water) were added (in triplicate) to the wells and allowed to incubate at room temperature for 2 h. After washing as above, 100 μl of goat anti-human IgG alkaline phosphatase conjuage (diluted 1:1000 in BSA–deionized water; for IgG analysis, incubated for 1 h) or goat anti-human IgE (diluted 1:1000 in BSA–deionized water; 1 h incubation, then wash, followed by rabbit anti-goat IgG alkaline phosphatase conjugate (diluted 1:1000 in BSA–deionized water), 1.25-h incubation) was added. The plates were washed and 100 μl of 0.6 mM *p*-nitrophenyl phosphate disodium (Sigma Chemical Co.) substrate solution, diluted in alkaline glycine buffer (0.05 M glycine and 0.5 mM magnesium chloride, pH 10.4), was added. After 30 min, the reactions were terminated with 50 μl of 2 N NaOH. Optical density at 410 nm with reference to 490 nm was read on an automated ELISA plate reader.

Statistical analysis

Analyses were performed using a microcomputer and a commercially available statistical analysis package (SPSS for Windows; SPSS Inc,. Chicago, IL). The significance of the prevalence of positive epicutaneous, pulmonary function and questionnaire results were evaluated by contingency table analyses. The ELISA results were evaluated for significance using Kruskal–Wallis's one-way ANOVA, followed by Mann–Whitney test; $P > 0.05$ was considered to be statistically significant.

RESULTS

The 36 participants were asked to respond to questions about work history, pre-existing medical conditions and symptoms related to their working with enzymes. Table

2 lists symptoms reported by the 36 participants and the frequency of work-related positive responses; 18% reported five or more symptoms. One worker reported experiencing 11 of the 13 symptoms listed in Table 2. There was a significantly increased ($P > 0.05$) prevalence of symptoms of respiratory, ocular and nasal irritation (itchy, watery eyes; sneezing; chest tightness; runny nose; cough). In addition, there was a significantly increased prevalence of flu-like symptoms, sweating and muscle aches. The prevalence of fever, sweatinng, shortness of breath, chills, rash and wheezing, although reported by 6–17% of the workers, was not statistically significant. Itchy, watery eyes, sneezing, chest tightness, runny nose and rash were combined to form an 'allergic symptom group' (ASG). A person was considered to meet the case definition for ASG if he or she responded positively to two or more ASG symptoms. In addition, sweating, muscle aches, fever, chills and flu-like sensation were grouped to form a 'flu-like symptom group' (FLSG). A person was considered to meet the case definition for FLSG if he or she responded positively to two or more FLSG symptoms. Fifteen persons (42%) had two or more ASG symptoms and nine persons (25%) had two or more FLSG symptoms. No association ($P < 0.05$) was found between meeting the case definition for either ASG or FLSG symptom groups and the following: job description, industrial hygiene personal exposure results, respiratory symptoms, years worked in enzyme manufacturing and years worked at the facility studied. Three persons reported having asthma and two of these reported having a physician diagnose their condition.

Thirty-eight workers were evaluated for allergic skin reactions. Skin test results from two workers were excluded from analysis because they did not develop a skin reaction when tested with histamine (the positive control). Three of the participants wished to participate exclusively in this phase of the medical evaluation. Table 3 lists the antigens applied and the number of workers with positive results (defined as a wheal of 3 mm or greater in the longest diameter). Eighteen persons (50%; $P > 0.05$) had positive skin reactions to commercial alkaline protease (CAP*Bl*, source organism B. licheniformis), eight persons reacted to amyloglucosidase (22%; $P > 0.05$; CAG*An*, source organism A. niger, commercial source), eight persons (22%; $P > 0.05$) reacted to AG*An* (source organism A. niger), six persons (17%; non-significant (NS)) had a positive reaction to α-amylase (CαA*Bl*, source organism B. licheniformis) and three persons (8%) reacted to αA1*Bs* (source organism B. subtilis) and αA*Bl* (source organism B. licheniformis). Two persons (6%; NS) had positive reactions to AP*Bl* (alkaline protease from B. licheniformis (commercial)) and CαA*Ao* (6%; NS) α-amylases from A. oryzae (commercial). One person had a positive reaction to αA2*Bs* (3%; NS; α-amylase from B. subtilis formulation 2). Nine persons were atopic as defined by reactions to two or more common aeroallergens ($P > 0.05$). Those with atopy had a greater likelihood ($P > 0.05$) of reacting positively to CAP*Bl* (commercial alkaline protease, source organism B. licheniformis) and commercial α-amylase (CαA*Bl*, source organism B. licheniformis).

Specific IgE and IgG antibodies to the antigens were evaluated using ELISA. Group mean antibody levels were determined for each antigen and compared with values obtained from three non-occupationally-exposed laboratory controls (immunology laboratory personnel with no known prior occupational exposure to enzymes). Mean IgG specific antibody levels were significantly higher ($P > 0.05$) for αA*Bl* (source organism B. licheniformis), αA1*Bs* (source organism B. subtilis), αA2*Bs* (source organism B. subtilis), CαA*Ao* (source organism A. oryzae), AG*An* (source organism A. niger), and CAG*An* (source organism A. niger) among exposed workers compared to controls (Fig. 1). Specific IgE antibodies for CαA*Ao* (source organism A. oryzae), AG*An* (source organism A. niger) and CAG*An* (source organism A. niger) were significantly higher ($P > 0.05$) among exposed workers than when compared to control sera (see Fig. 2).

To ascertain if elevated specific antibody levels were associated with certain job titles, job classification was

Table 2. Questionnaire results: most commonly reported symptoms

Symptom	Number reporting	Percentage ($N = 36$)
Itchty, watery eyes	13	36%*
Sneezing	12	33%*
Chest tightness	11	31%*
Muscle aches	11	31%*
Cough	10	28%*
Flu-like sensation	10	28%*
Runny nose	9	25%*
Sweating	8	22%*
Fever	6	17%
Shortness of breath	5	14%
Chills	4	11%
Rash	4	11%
Wheezing	2	6%

* $P > 0.05$.

Table 3. Epicutaneous test results

Antigen[a]	Employees with positive skin reaction	Participant percentage (36 workers tested)
CAP*Bl*	18*	50%
CαA*Bl*	6	17%
AG*An*	8*	22%
CAG*An*	8*	22%
αA1*Bs*	3	8%
αA*Bl*	3	8%
CαA*Ao*	2	6%
AP*Bl*	2	6%
αA2*Bs*	1	3%
Atopic	9*	25%

[a] CAP*Bl*, alkaline protease from B. licheniformis (commercial); CαA*Bl*, α-amylases from B. licheniformis (commercial); AG*An*, an amyloglucosidase from A. niger; CAG*An*, amyloglucosidase from A. niger (commercial); αA1*Bs*, α-amylase from B. subtilis formulation 1; αA*Bl*, α-amylase from B. licheniformis; CαA*Ao*, α-amylase from A. oryzae (commercial); AP*Bl*, alkaline protease derived from B. licheniformis; αA2*Bs*, α-amylase from B. subtilis formulation 2; atopic, positive skin test to two or more common aeroallergens. * $P > 0.05$.

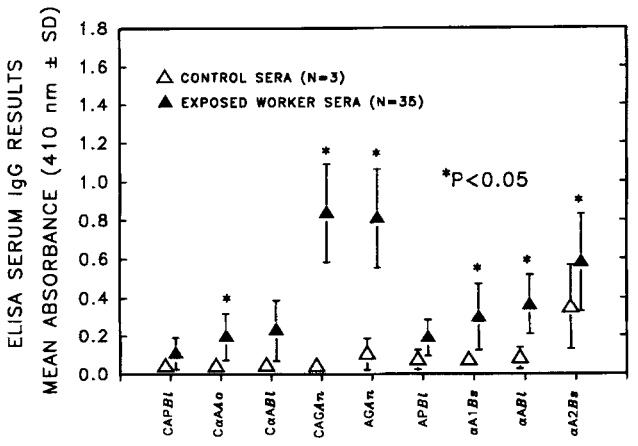

Figure 1. Specific IgE to process and commercial enzyme preparation (ELISA). Abbreviations are given in Table 1.

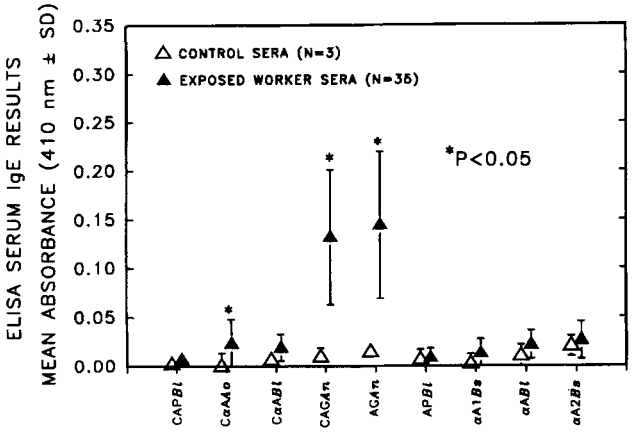

Figure 2. Specific IgG to process and commercial enzyme preparations (ELISA). Abbreviations are given in Table 1.

examined for the process workers and seven laboratory workers. A similar comparison could not be done for other job categories because they had too few participants in this study. We found no statistically significant association between the job categories evaluated and elevated IgG or IgE levels. In addition, there was no association observed between elevated specific antibody levels and years of employment, years worked in enzyme production, positive epicutaneous testing results, symptoms reported on questionnaire and personal exposure results.

Environmental measurements

Thirty-four of the 38 samples for alkaline protease, 23 of 24 samples for total protein and 24 of 26 samples for amyloglucosidase were below the LOQ for their respective assays (data not shown).

Pulmonary function:

Thirty-five persons (97%) completed pre-shift pulmonary function tests (PFTs). In addition, 12 process operators and foremen also completed post-shift PFTs. Three persons had a mild obstructive pattern and two persons had a mild restrictive pattern. None of the workers participating in both pre- and post-shift PFTs showed a significant decrease in FEV_1, or the ratio FEV_1/FVC. All decreases were less than 5% across the shift.

Each of the three workers with an obstructive pattern were process operators. A mild restrictive pattern was recorded for one process operator and one maintenance worker. Thus, work as a process worker entailed an increased risk for having mild obstructive or restrictive results on lung function testing compared to all other job classifications ($P > 0.05$).

Peak flow determination:

Thirty-five of 36 persons (97%) agreed to measure and record peak flow results for a 1-week period. Twenty-four workers had normal peak expiratory flow patterns (normal defined as less than a 20% difference between maximum and minimum PEFR on any day); the other 11 (31%) failed to chart results. One worker was noted to have experienced a 20% PEFR decline on the first day; however, this worker was one of the 11 employees who failed to chart for more than 1 day. The workers' peak flow results were also evaluated by calculating the amplitude percentage of the mean PEFR values (highest reading – lowest reading/mean for 24 h). This index was selected because previous studies have demonstrated that amplitude percentage of the mean provided the greatest separation between asthmatic and non-asthmatic populations (24,25). Individual results were considered to be positive if there was greater than a 15% variability in the amplitude percentage of the mean on at least 2 days. Three of 30 workers whose data could be analyzed by this method had positive results ($P < 0.05$).

DISCUSSION

This survey was done in response to reports of work-related symptoms (e.g. nasal congestion, itching eyes, shortness of breath), which suggested the possibility of IgE-mediated allergic reactions to enzymes or other workplace substances. Eighteen per cent of the 36 employees who completed the questionnaire reported five or more of the symptoms listed in Table 2. Fifteen persons reported two or more symptoms (e.g. itchy, watery eyes; chest tightness; runny nose), which, in many instances, can be mediated by IgE antibody. Testing for specific IgG antibodies in the workers' blood showed that mean IgG specific antibody levels were significantly higher for *αABl*, *αA1Bs*, *αA2Bs*, *CαAAo*, *AGAn* and *CAGAn* among exposed workers as a group than in non-exposed laboratory controls. Specific IgE antibodies for *CαAAo*, *AGAn* and *CAGAn* were significantly higher among workers than when compared to controls.

Twenty-two per cent of the workers had positive skin tests to *AGAn* and its commercial counterpart *CAGAn*. Fifty per cent had positive tests to alkaline protease *CAPBl*; however, only two individuals had positive skin tests to its process enzyme counterpart, *APBl*.

The high prevalence of positive skin tests to commercial alkaline protease (50%; *CAPBl*) without posi-

tive skin tests to its process enzyme counterpart, AP*Bl* (6%), in addition to the lack of specific IgE or IgG antibodies to CAP*Bl* or AP*Bl*, suggests that the response was one of non-specific irritation which was not controlled for in the design of the study. Alternatively, the relatively high (22%) prevalence of positive skin tests and significant levels of specific IgE and IgG antibodies to CAG*An* (commercial enzyme, source A. niger) and its process enzyme counterpart AG*An* (also from A. niger) leads to the speculation that CAP*Bl* is either contaminated or cross-reacts with A. niger or amylase glucosidase components. Other investigators have shown evidence of sensitization to A. niger in the biotechnology industry. At a biotechnology plant producing citric acid by fermentation of molasses with a strain of A. niger, 18 subjects were identified with work-related bronchospasm. Evidence of sensitization was demonstrated by positive skin prick tests and radioallergosorbent test (RAST) results using an extract of process A. niger culture fluid.[22]

A priori, we had presumed that enzyme-specific IgE levels would be associated with the observed statistically significant allergic symptoms found. However, in the present investigation, correlation analyses of symptoms, as well as other parameters, with levels of enzyme-specific IgE and IgG were non-significant ($P < 0.05$). This type of outcome is not unique, as we and others have reported positive skin tests and allergic symptoms even in the absence of detectable circulating antibodies with other occupational allergens.[28,29] Potential reasons for these types of observations include lowered levels of circulating antibody due to cellular binding, yielding reduced serum levels for in vitro analyses, while target organ-specific bound antibody is sufficient for elicitation of allergic reactions. In addition, the number of workers available in the present study may have been too low to determine weak associations between symptoms and antibody levels.

In conclusion, it appears that exposure to process enzymes in the setting we evaluated does lead to positive skin tests, increases in specific IgE and IgG antibody levels and instances of 'asthma- and flu-like' symptoms. Evaluation of all clinical and laboratory data suggests that components of A. niger and/or amyloglucosidase may be responsible for the observed effects. The greatest percentage of non-confounded positive skin tests and the highest levels of IgE- and IgG-specific antibodies were found to be associated with components of A. niger and amyloglucosidase from either process or commercial sources. Based on the results of these investigations it was recommended that employees be informed about the possibility of developing allergic reactions from exposure to the enzymes, as well as specific recommendations for engineering control and industrial hygiene practices described elsewhere.[26]

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