

Brief Report

IgE Sensitization to Bacterial and Fungal Biopesticides in a Cohort of Danish Greenhouse Workers: The BIOGART Study

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Background The use of biopesticides in agriculture may implicate new risks of work-related allergic reactions.

Methods Sera were tested from the BIOGART project, a longitudinal respiratory health study among >300 Danish greenhouse workers. IgE was measured by enzyme immunoassay (EIA) with extracts of biopesticide products containing *Bacillus thuringiensis* (BT) or *Verticillium lecanii* (Vert).

Results Many sera had detectable IgE to BT (23–29%) or Vert (9–21%). IgE titers from the 2- and 3-year follow-up ($n = 230$) were highly correlated, with discordant results in <15%. IgE titers to different BT, or to different *Verticillium* products were also significantly correlated (both $r > 0.70$), whereas IgE anti-BT and anti-*Verticillium* showed no correlation at all.

Conclusions Exposure to these microbial biopesticides may confer a risk of IgE-mediated sensitization. In future research there is a need to identify allergenic components in the preparations, perform studies on non-exposed controls and analyze the relation between sensitization and health parameters. Am. J. Ind. Med. 46:404–407, 2004.

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KEY WORDS: IgE; biopesticides; greenhouse workers; allergy

INTRODUCTION

The use of biologic pest control in agriculture has shown a rapid increase in the last few decades. Its beneficial effects, as an alternative for chemical pesticides, are obvious: not only would it markedly reduce the adverse ecological effects

of environmental pollution with chemical pesticides and the risks of pesticide residue exposure in the general public, it also would strongly diminish the hazards of occupational exposure to toxic pesticides and fungicides among agricultural workers. The most intensive use of biologic pest control takes place in greenhouses and horticulture, where there is a high susceptibility to plant diseases and insect pests due to the high crop density and specific climate conditions.

One of the (many) strategies in biologic pest control involves the use of insect pathogens: bacterial or fungal micro-organisms that more or less specifically infect and kill certain taxonomic groups of harmful insects like white flies or lice, while being harmless to most other invertebrates. A typical example is *Bacillus thuringiensis* (BT): a bacterium producing a potent enterotoxin that is mainly released in the gut of insects that have swallowed the BT bacteria [Schnepf et al., 1998]. An analogous fungal insect pathogen is *Verticillium lecanii* (Vert), which is mainly used to prevent and combat crop damage caused by aphids [Askary et al., 1999].

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The working mechanism of these agents, however, requires active dispersion of relatively large amounts of living or viable bacterial or fungal spores. The commercially available bacterial or fungal biopesticides are usually supplied as dried powders with a high microbial cell content, or as concentrated suspensions. Before application, suspensions are prepared or diluted in water in simple open buckets or other vessels from which the diluted suspensions are dispersed via a tubing system and aerosolized in the greenhouse. While the actual dispersal can be done in the absence of any workers in the greenhouse, exposure may occur after re-entry as dermal exposure when touching plants or other surfaces, and inhalation either of microbial cells that may remain airborne for prolonged periods, or of cells made airborne by the workers' activities.

So far, neither exposure levels nor the potentially associated health effects of biopesticide use have been systematically investigated. Possible adverse health effects could be either 'non-specific' airway inflammation, or specific type I, III, or IV allergic inflammation caused by allergen-specific sensitization. One study in the US has reported type I sensitization in a relatively small group of BT-exposed agricultural workers, as measured by skin prick tests and IgE serology [Bernstein et al., 1999], while no effects have as yet been reported for *Verticillium* use. In this study, the prevalence of specific IgE sensitization to extracts of commercially available preparations of BT and *Verticillium* was assessed among Danish greenhouse workers.

MATERIALS AND METHODS

Population

The serologic study was performed as part of the BIOGART study [Larsen et al., 2002]: a longitudinal epidemiologic study in a cohort of >300 Danish greenhouse workers, with a specific focus on respiratory health, including the prevalence and incidence of common and work-related respiratory symptoms, lung function status and changes, and development of bronchial hyper-responsiveness. As part of the study, venous blood samples have been taken at baseline and after 1, 2, and 3 years of follow-up, to measure total IgE and IgE to specific allergens, and to assess IgE sensitization to potential work-related allergens, particularly bio-pesticide components. This report contains the results for the 2nd year of follow-up, for which data was available, from 216 women (mean age 36.6 ± 10.2 yr) and 113 men (aged 36.3 ± 11.3 yr).

IgE Serology

Sera were tested for the presence of IgE to extracts of 8–10 different bio-pesticide preparations, including two products containing spores of *Verticillium* (Mycotal[®] and

Vertalec[®]) and two BT-based products (Bactimos[®] and Vectobac[®]). These biopesticides will be further designated as Vert-M, Vert-V, BT-B, and BT-V, respectively. Other biopesticides included in the study were products containing spores of *Trichoderma harzianum* (Supresivit[®]) or *Paecilomyces spp.* (Preferal[®]) and other BT preparations. According to the responses to yearly questionnaires on exposure, these products were much less used in the participating greenhouse companies, and accordingly, sensitization was rare. We, therefore, here only report the IgE anti-Vert and anti-BT reactions.

Extracts from dry powders (Vert-M and Vert-V) were made essentially as described earlier for the preparation of, e.g. wheat or potato antigen/allergen preparations [Houba et al., 1996; Zock et al., 1996]: 5% (w/v) suspensions were incubated overnight at room temperature in phosphate-buffered saline (PBS) containing 0.02% sodium azide, and the next day ultrasonicated for 15 min, followed by vigorous shaking for 30 min. Biopesticides supplied as suspension (BT-B and BT-V) were first centrifuged (30 min at 2,750g), and the supernatants were harvested and left overnight at room temperature. From the pellets, 3 g (wet weight) was mixed with 30 ml PBS-azide, and extracted overnight as described above for the 5% (w/w) powder suspensions, including ultrasonication and vigorous shaking for 30 min.

Insoluble material was removed by centrifugation (15 min; 2,100g) and filtration through paper filter and Millipore 0.45 μ m filters. The final extracts were extensively dialyzed against PBS-azide and stored in aliquots at -20°C . Protein concentrations in the extracts were determined with the BCA method (Pierce, Rockford, IL).

Serum IgE antibodies to biopesticides were assessed by a previously described enzyme immunoassay (EIA) method [Doekes et al., 1996]. Microtiter plates were coated overnight by incubation with the extracts in PBS-azide at 20 μ g protein per milliliter. After blocking with PBS containing 0.05% Tween-20 and 0.2% gelatin (PBTG), sera diluted 1/10 in PBTG were added and incubated for 2 hr at 37°C . After thorough washing of the plates with PBS-Tween, bound IgE was detected with a four-step procedure including mouse monoclonal anti-human IgE, biotinylated affinity-purified rabbit anti-mouse Ig, avidin-peroxidase, and finally *o*-phenylenediamine (OPD) plus H_2O_2 . The peroxidase reaction was stopped with 1 N HCl and the optical density read at 492 nm.

All sera were tested at a 1/10 dilution in duplicate wells for each test allergen/biopesticide extract, and in two non-coated wells to account for non-specific IgE binding to the plates, while in each plate also for each extract two wells were included with PBTG instead of serum ('no serum control'). The average OD_{492} values in allergen-coated wells were corrected both for the OD_{492} values in the non-coated wells, and for the OD_{492} of the no-serum controls. Sera were considered positive if the corrected OD_{492} was >0.05 [Doekes et al., 1996].

TABLE I. Positive Serum IgE Reactions to Extracts of Bio-Pesticides in the 2nd Year Follow-Up of the BIOGART Study (n = 329)

Biopesticide	Preparation	No. positive	
		sera	Percent
<i>Verticillium lecanii</i>	Vert-M (Mycotal [®])	28	8.5
	Vert-V (Vertalec [®])	58	17.6
	Vert-M and Vert-V	23	7.0
	Vert-M and/or Vert-V	63	19.1
<i>Bacillus thuringiensis</i>	BT-B (Bactimos [®])	78	23.7
	BT-V (Vectobac [®])	72	21.9
	BT-B and BT-V	60	18.2
	BT-B and/or BT-V	90	27.4

RESULTS

Table I gives a summary of the IgE EIA reactions for the four biopesticide preparations. High prevalences of sensitization were found for both *Verticillium*- and BT-based products: from 8.5% for *Verticillium*-M to 23.5% for BT-B. Positive reactions for the two *Verticillium* products were closely associated, with only 5 of the 23 IgE anti-Vert-M positive sera being negative on Vert-V, and 13 of the 35 IgE anti-Vert-V positive sera being negative on Vert-M (OR 35.0 and 95% CI 12.5–97.9). IgE anti-BT reactions showed an even more pronounced association, with more than 80% of sera reacting to either BT-B or BT-V also being positive on the other preparation (OR = 68.4 and 95% CI 30.3–145.3). In contrast, sensitization to *Verticillium* products on the one hand, and to BT on the other hand, showed no or only weak, non-significant associations, with ORs ranging from 1.4 to 1.8 (data not shown).

IgE reactions to extracts from products containing the same micro-organism were also quantitatively associated, as shown by the strong correlations between OD₄₉₂ values of 'double-positive' sera (Fig. 1a,b). These graphs further illustrate that the large majority of all positive reactions were relatively weak: only a few sera showed IgE anti-Vert-V and/or Vert-M reactions with OD₄₉₂ values around 1.0 or higher, and IgE anti-BT positive sera showed, with one exception, only OD₄₉₂ values <0.2.

From approximately two-thirds of the population a serum sample from the 3rd follow-up year was also available, and comparison of 2nd and 3rd year IgE serologic results could be made for 229–236 subjects for whom valid test results were available for both years. As shown by Table II, prevalences of positive IgE reactions in year 3 were very similar to those in year 2 (Table I), and a very close association was found between 2nd and 3rd year EIA results, with concordance levels of >90% and high positive and negative predictive values. Also highly significant quantitative correlations were found when the OD₄₉₂** of 2nd and 3rd year IgE EIA tests for individuals positive in both years, were plotted and compared (with Pearson's $r > 0.8$ for log-transformed OD₄₉₂ values).

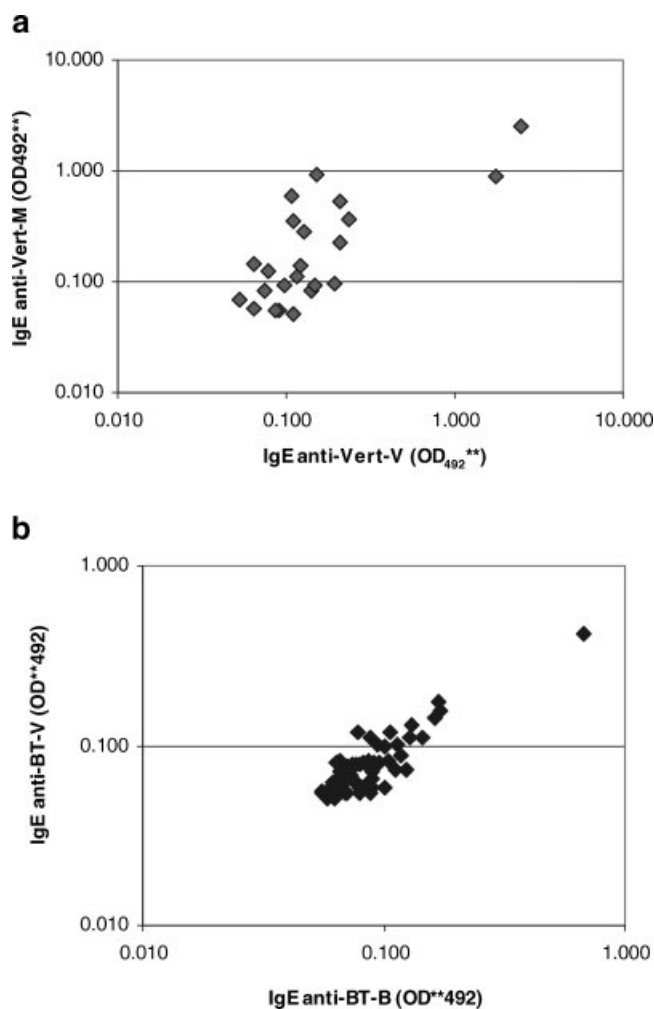


FIGURE 1. Correlation between OD₄₉₂ values (**) of positive IgE reactions to extracts of (a) two *Verticillium lecanii* (Vert) preparations: Vert-M (Mycotal[®]) and Vert-V (Vertalec[®]) (b) two *Bacillus thuringiensis* (BT) preparations: BT-B (Bactimos[®]) and BT-V (Vectobac[®]) (**) OD₄₉₂ values for 1/10 diluted sera, after correction for non-specific IgE binding to EIA plates, and for background reactions in 'no serum' control wells.

DISCUSSION

The results suggest that regular use of microbial biopesticides may be associated with a risk of biopesticide-specific IgE sensitization. There has previously been concern regarding the potentially negative health effects of exposure to BT, but most studies in this area have focused on the risk of infectious disease. In one study the prevalence of positive skin prick tests and of IgE reactions to BT extracts was investigated in a population of approximately 100 agricultural workers with estimated high or low levels of BT exposure [Bernstein et al., 1999]. While no evidence was found for work-related respiratory, possibly allergic symptoms, a significantly higher prevalence of positive SPTs and IgE tests was found among highly exposed farm workers. Present data are in line with these findings. In addition,

TABLE II. IgE Anti-Biopesticide Reactions: Comparison of 2nd and 3rd Year Follow-Up (N = 229–236)

Biopesticide	Positive sera in 3rd year (%)	Concordance between 2nd and 3rd year results (%)	PPV ^a (%)	NPV ^a (%)
Vert-M	22/230 (9.5)	96	80	97
Vert-V	49/232 (21.1)	90	77	93
BT-B	62/236 (26.3)	86	80	88
BT-V	47/229 (20.5)	88	73	91

^aPPV and NPV: Positive and negative predictive value of the 2nd year IgE test results.

another biopesticide, derived from *Verticillium* was identified as a possibly equally potent sensitizer.

Since the whole crude high molecular weight fractions extracted from the biopesticide products were used, the IgE reactions in the EIAs may be primarily directed against non-active carrier proteins, contaminants, etc. On the other hand, the strong correlations between IgE reactions against the two different BT products, and between the IgE reactions against two different *Verticillium* products, strongly suggest that in both cases the IgE antibodies are mainly directed against a genuine BT and genuine *Verticillium* allergenic HMW component. Nevertheless, the actual specificities of the IgE immune responses have to be studied in more detail, as well as their relation with exposure levels. At least, more extensive data series should be collected on IgE sensitization in non-exposed controls. Even while the IgE binding components may be genuine BT and *Verticillium* components, they could also be shared with some or many commonly found bacterial or fungal species, and in that case sensitization in the general population may be expected as well.

Another important issue may be the development of sensitization during repeated and/or continuous exposure at the workplace. We previously tested large numbers of sera from the first two years (years 0 and 1) of the cohort study, and found lower prevalences of IgE sensitization, while in the present series IgE EIA results for sera taken at year 2 and 3 of the follow-up showed a very close correlation (Table II). For three of the four products the prevalence of sensitization was slightly higher in year 3, which might mean that (a) while most sensitization takes place after 1–2 years of work-related exposure, some workers may be sensitized only after prolonged exposure; and (b) that an established specific IgE response may develop in subsequent years, especially when exposure continues.

It should be emphasized, however, that these reported results are of preliminary nature and that further analyses are required before definitive conclusions regarding the risks of occupational allergy can be drawn. While there was a relatively high prevalence of various airway and other symptoms in the studied cohort [Larsen et al., 2002], a first crude analyses did—just as in the earlier US study on BT sensitization [Bernstein et al., 1999]—not show a clear-cut direct association with a type I sensitization. A further analysis of

the biopesticide extracts, and particularly identification and characterization of the actual IgE binding components is an important and necessary next step to confirm the allergenicity of these agents.

In summary, these IgE serologic data may be a reason for concern that frequent use of microbial pesticides is a risk factor for occupational IgE-mediated allergic sensitization. Further studies focusing on both the specificity of these IgE reactions, and the association with work-related symptoms, are required to come to more definite conclusions.

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