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LEVELS OF OCHRATOXIN A AND IGG AGAINST CONIDIA OF *PENICILLIUM VERRUCOSUM* IN BLOOD SAMPLES FROM HEALTHY FARM WORKERS

Marit Aralt Skaug

Faculty of Health Studies, Hedmark University College, Elverum, Norway

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Abstract: Ochratoxin A (OTA) is a mycotoxin frequently found in human blood and milk samples in the colder climatic zones. In addition to dietary intake, exposure may occur by inhalation of toxin containing fungal conidia. The purpose of this work was to investigate the level of OTA in blood samples from farm workers and non-farm working controls, and to examine if serum levels of OTA were related to inhalatory exposure to conidia of *Penicillium verrucosum*, the main OTA producer in temperate climates. Blood samples from 210 participants were analysed for the presence of OTA and IgG antibodies against *P. verrucosum* conidia. The concentration of OTA was determined by HPLC (DL 10 ng/l), and the IgG level was determined by ELISA. All serum samples contained OTA (mean 397 ng/l, range 21–5534 ng/l). The OTA level in serum was unrelated to farm working, gender, age, and IgG level. The mean IgG level was significantly higher among farm workers than controls. Farm working, or increased inhalatory exposure to *P. verrucosum*, was not related to higher OTA serum levels. Inhalatory exposure to OTA from farm working seems to be of minor importance compared to dietary intake.

Address for correspondence: Marit Aralt Skaug, Associate Professor, PhD, Faculty of Health Studies, Hedmark University College (Høgskolen i Hedmark, HSE), 2418 Elverum, Norway. E-mail: marit.skaug@hse.hihm.no

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INTRODUCTION

Ochratoxin A (OTA) is a nephrotoxic, carcinogenic, teratogenic, and immunosuppressive mycotoxin produced by certain *Aspergillus* and *Penicillium* species. OTA can frequently be found in human blood and milk samples in Europe and Canada, indicating a continuous and widespread exposure to this mycotoxin [3, 9, 10, 15, 22, 25, 28, 31, 39]. However, human exposure to OTA is not uniform, but varies significantly between individuals and geographic regions. In previous studies, we have shown that the OTA levels were higher in human milk samples from women living in regions with extensive agriculture, than in areas with little or no agriculture [31, 34]. Moreover, a study from France reported higher levels of OTA in human blood samples from rural areas than from urban areas [5]. Heterogeneous OTA exposure can be due to individual and regional dietary differences. However, exposure to OTA can potentially also occur from inhalation of fungal conidia and agricultural dust [33]. Inhalatory exposure may represent an additional way of exposure to OTA, e.g. for farm workers who are commonly exposed to high concentrations of airborne organic dust and fungal conidia.

The purpose of this work was to investigate the levels of OTA in blood samples from farm workers and non-farm working controls, and to examine if serum levels of OTA were related to inhalatory exposure to conidia of *Penicillium verrucosum*, the major OTA producer in temperate climates.

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MATERIALS AND METHODS

Blood samples

A total of 106 farm workers and 104 controls (non-farm working blood donors) living in Hedmark County, Norway, were included in the study. One blood sample (8 ml) was drawn from each participant, from May–August 2000. The blood samples were collected in Hemogard Vacutainer plastic SST tubes, and serum was stored at -20ºC until subsequent IgG and OTA analyses. Data were obtained from all the participants on sex, age, smoking habits and occupation or type of farm work. The study was approved by The National Committee for Medical Research Ethics.

Determination of OTA

Extraction. OTA was extracted from serum according to the protocol described by Zimmerli and Dick [43]. A 1000 µl serum sample was mixed with 10 ml of a solution containing 33.7 ml 85% orthophosphoric acid, and 118 g NaCl per liter (pH without sodium chloride was 1.6). The mixture was shaken in a Vortex mixer for 1 min, 5 ml chloroform was added, and mixed for another 3 min. After centrifugation at 2500 g for 15 min, 4 ml of the organic phase was transferred to a 50 ml's flask. The chloroform extraction was repeated, and the combined chloroform extracts were evaporated to dryness under a stream of nitrogen gas (water bath at 30–40ºC).

Immunoaffinity cleanup. Immunoaffinity column was used for clean-up of the OTA extract (Ochraprep®, Rhône diagnostics). Before use, the immunoaffinity column was washed with 20 ml PBS (pH 7.4). The OTA residue was dissolved in 20 ml PBS (pH 7.4) with 5% acetonitrile, by ultrasonication for 15 min. The OTA extract was loaded and allowed to pass through the column at 2–3 ml/min. The immunoaffinity column was washed with 20 ml PBS pH 7.4 (flow 5 ml/min). OTA was eluated from the column using 1.5 ml acetic acid/methanol (2 : 98 v/v), followed by 1.5 ml methanol. The eluate was evaporated to dryness by nitrogen gas, and the residue was immediately dissolved in 300 µl of the HPLC mobile phase by ultrasonication for 15 min.

HPLC. The HPLC system consisted of a Merck Hitachi L-6200 A Intelligent Pump, fluorescence detector (Merck Hitachi F-1080), interface (Merck Hitachi D-6000), a Rheodyne manual injector, and chromatography software (model Hitachi D-6000 HPLC Manager). Separations were carried out on a Spherisorb S3ODS2 (C-18) column, 4.6 × 150 mm, with 3 µm particles.

The concentration of OTA was determined by HPLC ion-pair technique, at an alkaline pH and with fluorescence detection based on the method described by Breitholtz-Emanuelsson *et al.* [3]. A 50 µl aliquot of the sample extract was injected into the chromatograph. The mobile phase consisted of 10 mM tetrabutyl ammonium bromide in a methanol-potassium phosphate buffer (pH 7.5 and ionic strength 0.1) mixture. The ratio of methanol to potassium phosphate buffer was 51 : 49. Flow rate was 0.8 ml/min. The determinations were performed at 380 nm (excitation wavelength) and 450 nm (emission wavelength). The detection limit (signal-to-noise ratio of 3) was less than 10 ng/l. The mean recovery of OTA from spiked serum samples (range 190–400 ng/l) was 96.5% (n = 12, RSD = 8.8%). Results were not corrected for recovery.

Analytical quality control. Crystalline OTA (benzene free, from *Aspergillus ochraceus*) was purchased from Sigma Chemical CO. (St Louis, MO, USA). A solution of OTA (10 µg/ml in methanol) was calibrated spectrophotometrically at 333 nm, using the value of 6640 for the extinction coefficient [2]. The OTA solution was diluted to 10^{-8} M in methanol and stored at -20ºC. Working standard solutions for calibration were prepared every day by dilution of the 10^{-8} M OTA stock solution with HPLC mobile phase. For quantitation, peak heights were measured by a Merck Hitachi integrator. The calibration curves used for quantitation were calculated by the least-squares method.

At regular intervals, positive samples were qualitatively confirmed in two ways: 1) Sample extracts were analysed in duplicates, with a direct spiking of the second aliquot. This was performed by adding an amount of OTA stock solution directly to the injected aliquot. The chromatograms of the unspiked and the spiked sample extract were then compared. 2) Derivatization of OTA through methylation of the extracts with subsequent HPLC analysis was also used for qualitative confirmation of positive samples [43]: A 200 µl aliquot of the purified sample extract was evaporated to dryness, and the residue was dissolved in 2.5 ml methanol and 0.1 ml conc. HCl. The mixture was kept overnight at room temperature. After evaporating the mixture to dryness, the residue was dissolved in 200 µl mobile phase, and 50 µl of it was injected onto the HPLC column. Confirmation was based on the disappearance of the OTA peak, and the appearance of a new peak with approximately double retention time, corresponding to OTA methyl ester. No false positive samples were detected.

Determination of IgG

Antigen preparation. Isolates of *Penicillium verrucosum* IBC 5075 were grown on Sabouraud agar plates (135 mm in diameter), pH 5.9, and incubated at 25ºC in the dark for 2–3 weeks. The conidia from each plate were collected from the mycelial mat by washing with 3 × 10 ml sterile saline. The conidia suspension was homogenized by sonication for 60 min, and the homogenate was centrifuged (13000 rpm, 30 min). The supernatant was passed through 0.45 µm nitrocellulose filter, and dialyzed (Spectra/Por Membrane MWCO) for 24 h against distilled water. The dialyzed solution was freeze dried. The lyophilized antigen preparation was reconstituted in 10 mM phosphate-buffered saline (PBS pH 7.4), and filtered through 0.80 µm filter. The protein concentration was measured using the colorimetric method of *Lowry et al.* [21].

Table 1. Levels of OTA (ng/l) and IgG (relative absorbance) in serum. The difference in mean IgG between farm workers and controls is statistically significant (p = 0.030).

	No. of samples	Mean serum OTA, ng/l	Min.–Max. ng/l	Mean serum IgG	Min.–Max. Relative absorbance
Farm workers	106	371	21–2838	0.823	0–3.451
Controls	104	423	36–5534	0.653	0.016–2.571
Female	69	395	32–1923	0.839	0.049–2.650
Male	141	398	21–5534	0.694	0–3.451
Non-smokers	147	364	21–1740	0.779	0–3.451
Smokers	54	491	39–5534	0.653	0.049–1.802
Total	210	397	21–5534	0.741	0–3.451

ELISA procedure. Serum levels of IgG were measured by use of ELISA, with *Penicillium verrucosum* conidia sonicate as antigens. The test was performed on flat-bottomed microtiter plates with 96 wells (Falcon 3070). The antigen preparation was diluted in 0.01 M PBS pH 7.4 to give a protein concentration of 5 µg/ml. Sera and conjugate were diluted in PBS with 1% (w/v) BSA (bovine serum albumin, Sigma A-9647). Washing solution was PBS with 0.05 (v/v)% Tween 20. The conjugate used was swine anti-human IgG with alkaline phosphatase (Sigma A-1543). P-nitro-phenyl-phosphate (Sigma N-9389) diluted in glycinbuffer (1 mg/ml) pH 10.4 was used as substrate. The colour reaction was read in an automatic photometer (Multiscan), dual mode, at wavelength 405 nm versus 490 nm as reference wavelength.

The microtiter plate was coated with the antigen preparation. A volume of 150 µl was pipetted into each well, and the plate was sealed with adhesive tape and incubated for 3 hours at 37ºC. The coated plates were stored at 4ºC.

After the plates had been washed four times, 150 µl of diluted serum (1 : 25) was added in triplicates. PBS-BSA was used as negative control. The plates were incubated at 37ºC for 2 hours. After four washings, 150 µl anti-IgG conjugate diluted 1 : 20000 was added to each well. The plates were incubated at 37ºC for 2 h. After incubation, the wells were washed four times and 150 µl substrate (1 mg/ml) was added to each well and allowed to react for 30 min at 22ºC. The reaction was stopped by adding 25 µl 4 N NaOH. A control reference serum was included in every plate. The IgG level of sample sera was reported as mean absorbance at 405 nm relative to the absorbance of the reference serum.

Statistical analysis. Data were analysed by SPSS software program. Differences in mean OTA and IgG levels between groups were tested using *t*-test. Relationship between OTA and IgG was tested using Pearson's correlation test. Differences between the highest OTA exposed group and lower exposed individuals were analysed using the chi-squared test and *t*-test. A *p*-value < 0.05 was considered statistically significant.

RESULTS

All 210 serum samples analysed were found to contain OTA. The mean OTA concentration in serum was 397 ng/l (min. 21 – max. 5534 ng/l). Table 1 shows the mean OTA and IgG levels in serum. The mean OTA level in serum from farm workers (371 ng/l) was not significantly different from controls (423 ng/l). Overall, OTA level in serum was unrelated to type of farm working (dairy, poultry, swine farming), gender, and age. There was a non-significant trend towards higher levels of OTA in sera from smokers compared to non-smokers. To examine whether high levels of OTA in serum were related to specific characteristics, individuals with the highest OTA serum levels were compared with those with lower OTA levels. No differences could be found between high-exposed individuals, defined as the 95th percentile (OTA ≥ 866 ng/l) and those with lower OTA levels, regarding farm working, gender, age, IgG levels, or smoking.

The mean level of IgG against *Penicillium verrucosum* conidia in serum from farm workers was significantly higher as compared to control sera. The mean relative absorbance in the farm worker group was 0.823 (SD 0.637), compared to 0.653 (SD 0.457) in the control group (p = 0.030). Serum IgG level was otherwise not related to OTA level, type of farm working, smoking, gender, or age.

DISCUSSION

All the serum samples analysed in this study contained OTA, confirming a widespread and frequent human exposure to this mycotoxin. One hundred percent OTA positive samples have also been reported from other countries, e.g. Switzerland [43], and Sweden [38]. The overall mean level of OTA in serum (mean 397 ng/l, n = 210) was higher than previously reported from Norway. In a study of 202 blood donors living in the city of Oslo, the mean serum OTA level was 180 ng/l [38]. The difference in OTA levels between the two studies may reflect a higher OTA exposure in rural areas (Hedmark, present study) than in urban areas (Oslo), in accordance with results from previous investigations on

OTA levels in human blood [5] and milk [31]. However, the possibility of differences in OTA contamination due to annual variations cannot be ruled out. The blood samples from Oslo were collected in February 1998, whereas the blood samples in the present study were collected in May–August 2000. The serum levels of OTA vary significantly between individuals, and in some samples, exceptionally high levels of OTA have been found [10]. In a study of 163 serum samples from Switzerland, the OTA concentration range was from 60 to 6,021 ng/kg [43]. In a German study of 306 serum samples, the range of positive samples was from 100 to 14,400 ng/kg [2]. A high serum level of OTA is generally assumed to indicate a high exposure level. However, the serum level of OTA is also determined by toxicokinetic variables, in particular the renal clearance. Therefore, high serum levels of OTA in some individuals can possibly be due to impaired clearance of the toxin.

The main route of human exposure to OTA is through dietary intake of contaminated foods, e.g. cereals, bread, pork and poultry meat, coffee, beer [15, 16, 37, 40], wine, red grape-juice [44], and cow's milk [3, 32]. In addition to diet, exposure to OTA can possibly occur by inhalation of toxin containing conidia and airborne dust. In a recent study, conidia from *Penicillium verrucosum* were found to contain OTA levels on the order of 0.5 pg per conidium. Furthermore, the presence of OTA (0.2–70 µg/kg) was demonstrated in samples of airborne agricultural dust [33]. Animal experiments have shown that OTA is very efficiently absorbed from the respiratory system. When rats were given a solution of OTA by intratracheal tubes, 98% of the toxin entered the systemic circulation [4]. Conidia from *Penicillia* are usually small, less than 5 µm in diameter, and are therefore easily inhaled into the lungs. OTA associated with conidia, or other particles entering the respiratory system, can be absorbed by the respiratory epithelium and distributed to other sites. One case of human illness linked to inhalatory OTA exposure has been reported. A female farm worker suffered from acute renal failure after working for 8 hours in a granary, which had been closed for several months. Inhalation of *Aspergillus ochraceus* conidia containing OTA was implicated [6].

The present investigation was initiated to examine if exposure to airborne *Penicillium verrucosum* conidia leads to increased OTA serum levels. Increased levels of IgG antibodies against airborne antigens can be used as biomarkers of fungal exposure [18, 20, 41, 42]. Although *Aspergillus* and *Penicillium* species are common in the home environment, increased IgG antibody levels to these fungi have been found in exposed populations compared to non-occupationally exposed populations, suggesting that elevated IgG levels can be ascribed to occupational exposure to these fungi [1, 12, 18, 27]. The results of this study show that levels of IgG against *Penicillium verrucosum* conidia were significantly higher in farm workers than in non-farm working controls, indicating higher exposure of farm workers, as was to be expected. Farm workers are frequently exposed to high concentrations of airborne organic dust and fungal conidia, especially when handling plant materials [7]. *Penicillium verrucosum*, the main OTA producer in Northern Europe [8, 14, 19, 26], is commonly found on stored cereals [11, 23, 24, 29], forages, grain feeds [14, 36], and in air samples [17, 36].

The results show a higher degree of exposure to *Penicillium verrucosum* conidia among farm workers than controls, but the elevated exposure was not related to higher OTA serum levels.

CONCLUSION

Farm working, or increased inhalatory exposure to *Penicillium verrucosum* conidia, was not associated with increased OTA exposure. This indicates that inhalatory exposure to OTA from farm working is of minor importance compared to dietary intake. Higher OTA levels found in sera from rural populations compared to urban populations seems to be caused by differences in dietary habits rather than farm working.

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