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Changes in serum markers indicative of health effects in vineyard workers following exposure to the fungicide mancozeb: an Italian study

CLAUDIO COLOSIO[1], SILVIA FUSTINONI[2], EMANUELA CORSINI[3], CRISTINA BOSETTI[4], SARAH BIRINDELLI[5], DAISY BOERS[6], LAURA CAMPO[2], CARLO LA VECCHIA[4,7], JYRKI LIESIVUORI[8,9], SIRPA PENNANEN[8], TATJANA VERGIEVA[10], LUDOVIC G. P. M. VAN AMELSVOORT[6], PETER STEERENBERG[11], GERARD M. H. SWAEN[6], CHRISTO ZAIKOV[10], & HENK VAN LOVEREN[11,12]

[1]*Department of Occupational and Environmental Health of the University of Milan, S. Paolo Hospital Unit, Milan, Italy,* [2]*Department of Occupational and Environmental Health, University of Milan and Fondazione IRCCS Ospedale Maggiore Policlinico, Mangiagalli e Regina Elena, Milan, Italy,* [3]*Laboratory of Toxicology, Department of Pharmacological Sciences, University of Milan, Milan, Italy,* [4]*Istituto di Ricerche Farmacologiche 'Mario Negri', Milan, Italy,* [5]*International Centre for Pesticides and Health Risk Prevention, University Hospital L. Sacco, Milan, Italy,* [6]*Department of Epidemiology, Maastricht University, the Netherlands,* [7]*Istituto di Statistica Medica e Biometria, University of Milan, Italy,* [8]*Finnish Institute of Occupational Health, Kuopio, Finland,* [9]*Department of Pharmacology, Drug Development and Therapeutics, University of Turku, Finland,* [10]*National Centre of Public Health Protection, Sofia, Bulgaria,* [11]*National Institute of Public Health and the Environment, Bilthoven, the Netherlands and* [12]*Department of Health Risk Analysis and Toxicology, Maastricht University, the Netherlands*

Abstract

The aim of this study was to investigate the health effects induced by exposure to the fungicide mancozeb in Italian vineyard workers. Ninety-three Italian subjects entered the study – 48 vine-growers intermittently exposed to mancozeb and 45 healthy controls. The subjects were investigated three times: before the seasonal application of pesticides (T0), 30 days after the beginning of the application period (T30), and 45 days after T0 (T45). At T0 the comparison between agricultural workers and controls showed a higher prevalence of cold or flu symptoms, a statistically significant lower percentage of monocytes, higher absolute count of T lymphocytes, CD4 and natural killer cells, and lower plasma levels of IgA and IgM in workers. Such differences were not confirmed at T30 and T45. In fact at T30 in exposed workers, besides a significant increase of urinary ethylenethiourea, confirming mancozeb exposure, T lymphocytes, CD4 and natural killer cells, IgA and IgM returned to values comparable to those observed in controls. Moreover, no other differences in clinical signs, haematological, and

Correspondence: Claudio Colosio, Department of Occupational and Environmental Health of the University of Milan, S. Paolo Hospital Unit, Via Di Rudinì 8, 20142 Milan, Italy. Tel: +39 0250323036. Fax: +39 0250323305. E-mail: claudio.colosio@unimi.it

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immune parameters, such as the immune functional capability evaluated as a response to hepatitis B vaccination, was observed. Altogether the differences between exposed and controls were not consistently correlated to any clinical impairment and suggest that the seasonal application of mancozeb does not pose a significant health risk to exposed subjects.

Keywords: *Ethylenebisdithiocarbamate fungicides (EBDCs), mancozeb, immunotoxicty, humans.*

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Introduction

Due to their short environmental persistence and low acute toxicity, ethylenebisdithiocarbamate fungicides (EBDCs) are used worldwide (Maroni et al. 2000). As a consequence, most of the human population is potentially exposed to these compounds and their residues, either in occupational settings (industry and agriculture) or in the living environment (Colosio et al. 2006). While the low acute toxicity of EBDCs is well known, data on possible effects of prolonged, low-dose exposure are lacking.

In the past decades, laboratory studies supporting evidence of an immunomodulatory effect of dithiocarbamates and EBDCs has been collected (Renoux et al. 1988, Lombardi et al. 1991, Padgett et al. 1992, Pruett et al. 1992). Sodium ethylenebisdithiocarbamate (nabam) has been demonstrated to be a potent *in vivo* immunomodulator, influencing maturation and activation of T cells, natural killer (NK) cells and immunoglobulin G (IgG) secretion, and prolonging immunological memory (Renoux & Renoux 1980, Renoux et al. 1988, Padgett et al. 1992, Pruett et al. 1992). Zinc ethylenebisdithiocarbamate (zineb) was found to be devoid of immunoenhancing influence in response to T-cell mitogens, and it exerted a cytotoxic effect on spleen lymphocytes (Renoux et al. 1988). Sodium methyldithiocarbamate has also been reported to cause significant immunosuppression in mice following *in vivo* exposure (Padgett et al. 1992, Pruett et al. 1992). Some data suggest that in humans EBDCs and their main metabolite, ethylenetiourea (ETU) might cause disorders based on enhancement of the immune response, such as allergic contact dermatitis (Matsushita et al. 1976, Kleibl & Rackova 1980, Bruze & Fregert 1983, Higo et al. 1996, Iliev & Elsner 1997). Altogether, available data suggest that the only immune effect clearly observed in EBDC-exposed workers is sensitization, while there is insufficient evidence of any other kind of immunotoxic effect consequent to occupational or environmental exposures.

Studies carried out in workers occupationally exposed to the zinc manganese ethylenebisdithiocarbamate (mancozeb) showed an increase in serum IgG, IgE and β2-macroglobulin (Jablonicka et al. 1989), suggesting an immunostimulating effect. An increase in T-cell proliferative responses to mitogens was observed in industry workers occupationally exposed to mancozeb (Colosio et al. 1996), also confirmed in a group of agricultural workers involved in mancozeb application in vineyards (Corsini et al. 2005). The main limit of these studies is the uncertainty of the clinical implications of these findings. In particular, it is still unclear whether these effects can be considered only adaptive, or potentially able to evolve into disease, such as allergy or autoimmunity. For these reasons mancozeb was included in the group of pesticides to be tested in a multicentric European study (EUROPIT) sponsored by the European Union. The aim of this project was to assess the

immunotoxic potential of selected pesticides in occupationally exposed workers. In the present paper we report the results of the Italian study, in which the effects of mancozeb exposure on the haematological and immune systems of vineyard workers were investigated.

Materials and methods

Study population

The study was carried out in two rural areas located in Northern Italy, one in Pavia province (region of Lombardy) and the second in Trento province (region of Trentino-Alto Adige), between March and June of 2002 and 2003, i.e. immediately before the spraying season and the end of the seasonal use of mancozeb. Ninety-three subjects entered the study: 48 vineyard workers intermittently exposed to mancozeb (14 from Pavia and 34 from Trento) and 45 control subjects (11 from Pavia and 34 from Trento) not exposed to known sources of pesticides. The workers from the region of Lombardy were the total workforce exposed to pesticides in 11 small vineyards selected in the territory by the local Occupational Health Unit of the Public Health System, while the whole group of workers selected in the Trento Province was engaged in a single, large fruit production unit.

Exclusion criteria for the subjects included: age less than 18 or greater than 60 years, presence of any major confounding factors such as current use of medications known to affect the immune system (i.e. immunostimulators, immunosuppressive compounds such as cytostatics or steroids, recent vaccinations) or subjects suffering from malignancies, or recovering from major trauma and burns.

In agricultural workers, pesticide exposure occurred during the preparation of the pesticide mixture and its application in vineyards ($n = 31$), or during activities involving workers' entry in the crops after pesticide application (re-entry activities) ($n = 17$).

Subjects were selected according to the guidelines of the Italian Health authorities and to the Declaration of Helsinki principles. All subjects agreed to take part in the investigation and signed an informed consent.

Study protocol

Recent exposure to mancozeb was assessed by measuring urinary ETU, a major metabolite of mancozeb in humans. The potential immunotoxic effects of mancozeb were investigated using several blood cellular and serum parameters such as complete and differential blood cell counts, lymphocyte subpopulations, α1-glycoproteins, erythrocyte sedimentation rate (ESR), complement 3 and 4 fractions (C3, C4), antinuclear (ANA), antismooth muscle (SMA) and antimitochondrial (AMA) autoantibodies, and serum immunoglobulins (IgG1, IgG4, IgM, IgA, and total and specific IgE to most common allergens).

The immune functional capability was evaluated by determining the response to hepatitis B vaccination in previously non-immunized subjects.

General health conditions and specific clinical signs of impairment of the immune system were investigated both by physical examinations and by self-administered questionnaires.

For exposed workers, three time points were investigated. The first time point was just before the beginning of the seasonal use of mancozeb, between the end of March and the end of April (T0). At this time point, a complete set of tests was applied: questionnaire, determination of urinary ETU, blood cellular and serum parameters, antibody titre and physical examination. Moreover, the antihepatitis B vaccination was first administered to subjects who had not been immunized previously, and who agreed to be vaccinated. A second time point (T30), was investigated after an exposure period of about 30 days, during which time mancozeb was intermittently applied, with patterns depending mostly on the local weather and the crop conditions. At this second time point the following tests were applied: urinary ETU level, blood cellular and serum parameters, and physical examination. Information on pesticide use during the exposure period and on the possible onset of exposure-related symptoms was collected with a second version of the questionnaire; in addition, a second dose of vaccine was administered. At the third time point, about 40–45 days after the administration of the first, and 10–15 days after the administration of the second dose of vaccine (T45), the immune system response to hepatitis B vaccination was investigated.

For controls, just two time points were investigated: T0 and T45. At these time points the same protocols used for agricultural workers were applied. At T30 they received the second dose of vaccine, and a third booster vaccination 5 months after the second dose. This was not part of the study, but was necessary to complete the vaccination procedure.

The data obtained were used to make comparisons between agriculture workers and controls or, within agricultural workers, between different time points. In particular the comparison at T0 between exposed subjects and controls was to investigate possible immune effects of exposure lasting over time. The comparison between T30 and T0 in agricultural workers was to investigate the effects of short-term exposure to mancozeb. Finally, the comparison between the two groups at T45 was to evaluate a possible impairment of the immunization capacity, which may be considered either a short- or a long-term effect of exposure.

Questionnaires

The questionnaires included personal questions on demographic factors, presence of diseases, with particular attention to allergic, infectious and autoimmune diseases, vaccinations, use of drugs, alcohol intake and tobacco smoking. Furthermore, job information was covered by questions on job title, company, duration of employment and, for exposed subjects, type of pesticides used.

Physical examination

A standardized protocol for physical examination was prepared and applied to examine the subjects. For each organ or system all the possible changes together with their severity, were taken into account. The results of the physical examination were registered on a form. In particular, at T30, any possible effect related to pesticide exposure was noted, with emphasis on mild and pre-clinical changes. The last paragraph of the form was for the comments and conclusions of the occupational health physician, in free text.

Biological monitoring

Sample collection. Urine spot samples were collected in the morning, as the second void of the day. Because of the relatively short half-life of urinary ETU in humans, samples from exposed subjects were collected within 16 h after the end of the exposure. For collection, 15 ml polyethylene tubes shielded from light by aluminium foil were used. Samples were chilled, and delivered to the laboratory within 24 h. In the laboratory, the samples were kept at -20°C until analysis.

Blood samples for the definition of cellular and serum parameters and some functional parameters were collected by vein puncture. Samples were kept at room temperature and delivered to the laboratory on the day of collection. Cellular parameters were determined within 24 h, while sera, obtained by centrifugation, were stored at -80°C prior to analyses.

Exposure assessment. ETU was isolated from urine by liquid/liquid extraction using dichloromethane, reacted to form a bis(*tert*-butyldimethylsilyl) derivative and analyzed via gas chromatography-mass spectrometry as previously reported (Fustinoni et al. 2005). Analytical detection limit is 0.6 $\mu g\ l^{-1}$.

Urinary ETU was adjusted for urinary creatinine. Creatinine was determined using Jaffe's colorimetric method. In brief, creatinine was reacted with an aqueous solution of picrate (1% p/v) in NaOH (2.5 M). The quantification was based on the absorbance of the picrate complex at 512 nm, determined using a UV-VIS spectrophotometer. Levels ranging between 0.3 and 3.0 $g\ l^{-1}$ were considered acceptable (WHO 1996). Urinary ETU is expressed as $\mu g\ g^{-1}$ creatinine.

Immune system investigation – serum and cellular investigation. The determination of complete and differential blood cell counts was made by light microscopy. Complete lymphocyte subpopulations, namely T lymphocytes, CD4 (helper/inducer), CD8 (suppressor/cytotoxic), B lymphocytes and NK cells were assessed by flow cytometric analysis as previously described (Corsini et al. 2005) Briefly, heparinized peripheral blood (50 μl) was stained with an appropriate concentration of fluorescein- or phycoerithrin-conjugated monoclonal antibodies. Results were expressed either as a percentage or as an absolute number of positive cells.

The determination of α1-glycoproteins, ESR, C3, C4, ANA, SMA and AMA were determined by immunokinetic nephelometry indirect immunofluorescence assay. The determination of serum immunoglobulins (IgG1, IgG4, IgM, IgA, total and specific IgE to most common allergens) was performed in sera by specific sandwich analysis. Antibodies against human IgG1 and IgG4 and relative standards were obtained from Calbiochem (San Diego, CA, USA), while antibodies against IgA and IgM and relative standards were from Sigma (St Louis, MI, USA). Briefly, for IgG1 and IgM high-binding 96-well plates (Greiner, Frickenhausen, Germany) were coated overnight at 4°C with 2 $\mu g\ ml^{-1}$ of monoclonal antibodies diluted in phosphate buffered saline (PBS), while for IgA and IgG4 plates were coated with 4 $\mu g\ ml^{-1}$ of monoclonal antibodies. Plates were washed three times with PBS containing 0.05% Tween 20, then diluted serum (0.1 ml) or standards were added in duplicate for 2 h at room temperature. After three washes, 0.1 ml of specific secondary antibodies alkaline phosphatase-conjugated diluted 1:2000 (for IgA and IgM) or 1:5000 (for IgG1 and IgG4) in PBS were added for 1 h at room temperature. Para-nitrophenyl phosphate (Sigma) was used as a substrate, and the absorbance was measured at 405 mm.

The IgE concentration against the following allergens was determined: cat, dog, house dust mite, mixture of grass pollen, mixture of tree pollen, mixture of herb pollen, mixture of fungi. The IgE concentration was determined in sera by immunofluorescence, using a commercially available immnunoCap method (Phadia B.V., Nieuwegein, the Netherlands). Immunoglobulin results are expressed as mg ml^{-1}.

Antihepatitis B titre. The antibody titre was assessed in sera using a commercially available kit (Enzygnost anti-HBs II, Dade Boehring, Marburg, Germany). Results are expressed as UI l^{-1}.

Statistical analysis

The relationship of mancozeb exposure and immunosystem markers was assessed by comparisons between exposed and controls and within exposed, comparing parameters assessed at T0 and T30 or T45. A value corresponding to half of the detection limit was assigned to ETU measurements below analytical detection. Giving the small sample size and the skewedness of the data, the tests of significance were based on non-parametric techniques, namely the Mann–Whitney U test for comparing independent samples and the Wilcoxon's test for comparing paired data. A p value of 0.05 was considered statistically significant. The analyses were carried out using the SAS software (SAS Institute Inc., 1989).

Results

Relevant personal information regarding the investigated subjects, including gender, age, body mass index, job title, education and area of residence or reported symptoms are summarized in Table I. The statistically significant differences between exposed and controls are represented by education (higher in controls) and area of residence (the proportion of exposed subjects living in rural areas was higher in the exposed group). When health symptoms were investigated the T0 questionnaire indicated the presence of a statistically significant greater proportion of subjects reporting cold or flu in the previous 4 weeks in exposed subjects compared with controls (27% vs. 9%). In contrast, the proportion of subjects who reported hay fever, allergic contact dermatitis, skin rash or bleeding gums, was slightly higher in controls than in the exposed subjects, as well as the proportion of subjects who ever had asthma, even diagnosed by a physician, even if none of these differences was statistically significant.

Additional information was taken from the questionnaire: in the majority of agricultural workers at T30, the use of pesticides other than mancozeb was reported. The active ingredients of the applied formulations were sulphur (used by 20 subjects), dimetomorph (nine subjects), quinoxyfen (six subjects), penconazole (five subjects), copper and metyram (three subjects each). None of these active ingredients is suspected or known to act as an immune modulator. As for mancozeb, this active ingredient was used according to the label instructions with a use rate of 1.4–1.6 kg of active substance per hectare.

The drug intake during the previous 6 months, including the investigated period, did not show a statistically significant difference between the investigated groups either taking into account the proportion of subjects taking medication or the type of

Table I. Summary of characteristics of study subjects divided according to job title.

	Exposed ($n=48$)	Controls ($n=45$)	p-value for comparison exposed vs. controls
Males, n (%)	37 (77%)	33 (73%)	n.s.
Females, n (%)	11 (23%)	12 (27%)	
Age (years), mean (SD)	42 (11)	41 (11)	n.s.
Body mass index (kg m^{-2}), mean (SD)	25.2 (2.7)	24.1 (3.1)	n.s.
Job title, n (%)			
Pesticide application and mixing and loading	26 (54)	–	
Re-entry workers	22 (46)	–	
Clerks, teachers and students	–	18 (41)	
Healthcare workers	–	20 (46)	
Others (retired, industry workers)	–	7 (13)	
Education, n (%)			
Low	25 (54)	6 (14)	<0.001
Intermediate	21 (46)	18 (41)	
High	0 (0)	20 (45)	
Area of residence, n (%)			
Urban	1 (2)	16 (36)	<0.001
Rural	45 (98)	29 (64)	
Size of town, median (min–max)	1900 (4–38 000)	2150 (100–106 000)	n.s.
Reported symptoms, n (%)			
Cold in the previous 4 months	30 (64)	24 (55)	n.s.
Flu in the previous 4 months	15 (33)	8 (19)	n.s.
Gastroenteritis in the previous 4 months	7 (15)	5 (11)	n.s.
Cold or flu symptoms in the previous 4 weeks	13 (27)	4 (9)	<0.05
Respiratory infections in the previous 4 weeks	6 (13)	2 (5)	n.s.
Skin infections in the previous 4 weeks	4 (8)	3 (7)	n.s.
Hay fever or allergic symptoms in the previous 4 weeks	3 (6)	6 (14)	n.s.
Skin rash in the previous 4 weeks	1 (2)	3 (7)	n.s.
Bleeding gums in the previous 4 weeks	3 (6)	6 (14)	n.s.
Cold sores or fever blisters in the previous 4 weeks	4 (9)	3 (7)	n.s.
Arthritis symptoms in the previous 4 weeks	13 (28)	8 (19)	n.s.
Other illnesses in the previous 4 weeks	2 (4)	4 (9)	n.s.
Ever had asthma	2 (4)	4 (9)	n.s.
Asthma diagnosed by a physician	2 (4)	3 (7)	n.s.
Currently taking any medicines for asthma	1 (2)	1 (2)	n.s.
Ever had an itchy rash	1 (2)	5 (12)	n.s.

n.s., not significant or $p>0.05$.

Table II. Urinary ethylenethiourea in subjects divided according to job title and geographical area of recruitment.

	Exposed		$p^{\star}$ for comparison exposed T0 vs. exposed T30	Controls	p° for comparison exposed T0 vs. controls T0
	T0	T30		T0	
All subjects					
Mean (SD)	1.8 (5.3)	14.9 (13.0)	<0.001	1.3 (1.5)	n.s.
Median (min–max)	<LOD (<LOD–37.1)	11.8 (<LOD–62.5)		0.5 (<LOD–8.1)	
Subjects from Pavia					
Mean (SD)	4.2 (9.7)	23.5 (17.2)	<0.001	1.4 (2.3)	n.s.
Median (min–max)	<LOD (<LOD–37.1)	25.9 (3.1–62.5)		<LOD (<LOD–8.1)	
Subjects from Trento					
Mean (SD)	<LOD (0.6)	11.3 (9.0)	<0.001	<LOD (1.2)	n.s.
Median (min–max)	<LOD (<LOD–3.1)	9.9 (<LOD–42.8)		0.7 (<LOD–5.1)	
p# for comparison Pavia vs. Trento	n.s.	<0.05		n.s.	

LOD, limit of detection; n.s., not significant or $p > 0.05$.

drug assumed. Also the physical examination did not indicate any statistically significant differences between groups.

The levels of urinary ETU in the investigated subjects divided by job title and then by geographical area of recruitment, are shown in Table II. The results confirm that during the observed period vineyard workers were exposed to mancozeb, as a significant increase in ETU excretion was observed after the exposure period. At T0, in a significant proportion of both exposed subjects and controls a low but detectable amount of ETU was found, but no statistically significant differences between agricultural workers and controls were observed. Comparing agriculture workers from the two different geographical areas of recruitment we noted that ETU levels in subjects from Trento were significantly lower than those observed in subjects from Pavia.

In Table III, the results of the blood cellular parameters are reported. At T0, the agricultural workers showed slight differences in comparison with control subjects in some immune parameters, including a lower percentage of monocytes, a higher absolute number of T lymphocytes, CD4 and NK cells. Within agricultural workers following exposure, a tendency toward a reduction in the percentage of monocytes, and the number of T lymphocytes, CD4 and NK cells was observed.

In Table IV the immunoglobulin levels and other serum parameters are reported. While most parameters were determined in all the investigated subjects, some missing values were present for α1-glycoprotein and ESR at T0 either in exposed or in controls. This was due to problems during sample collection, so that coagulation of some blood samples occurred. At T0, the agricultural workers showed lower plasma levels of IgA and IgM in comparison with controls. Within agricultural workers a significant increase of serum concentrations of IgA and IgM was observed after exposure. Concerning the other serum parameters, no biologically relevant differences were observed between groups or at different times. Moreover, no statistically significant differences were observed among groups in the total or specific IgE levels (data not shown).

As for the functional investigation, the number of subjects vaccinated during the study was very small, as the majority of both exposed subjects and controls had already been immunized against hepatitis B or refused to be vaccinated. As a consequence only 16 exposed subjects and nine controls were available for this test. Nevertheless, the comparison between exposed workers and controls failed to show any statistically significant difference, taking into account either the percentage of immunized subjects or considering the antibody titre.

Discussion

The aim of this study was to investigate the effects of mancozeb on several immune parameters in agricultural workers intermittently exposed to the fungicide. In particular short-term and long-term effects were investigated comparing workers and controls before and/or after the seasonal application of the fungicide.

The significant increase of urinary ETU in agricultural workers following exposure confirmed that absorption of mancozeb took place during application; in fact, postexposure urinary ETU exceeded the reference value of 5 $\mu g\ g^{-1}$ creatinine, tentatively defined for the general population (Colosio et al. 2006), by about one order of magnitude. The short half-life of ethylenedithiocarbamates in humans limits the use

Table III. Blood cellular parameters in study subjects divided according to job title.

	Exposed			Controls	
	T0	T30		T0	
	Median (min–max)	Median (min–max)	p⋆ for comparison exposed T0 vs. exposed T30	Median (min–max)	p° for comparison exposed T0 vs. controls T0
Erythrocyte (10^6 mm^{-3})	4.92 (3.86–6.56)	4.94 (4.11–6.21)	n.s.	4.94 (3.78–6.06)	n.s.
Haemoglobin (mmol l^{-1})	15.1 (7.5–17.7)	14.9 (10.4–17.0)	n.s	14.7 (11.7–16.8)	n.s.
Haematocrit (%)	43.7 (23.8–52.0)	44.2 (32.7–51.9)	n.s.	42.7 (35.3–49.3)	n.s.
Thrombocyte (10^9 l^{-1})	227 (60–418)	225 (136–353)	n.s.	227 (119–353)	n.s.
Leucocyte (10^3 mm^{-3})	6.28 (2.92–10.43)	5.94 (3.22–8.82)	n.s.	5.67 (3.86–14.29)	n.s.
Granulocytes (%)	55.1 (42.2–79.4)	55.3 (41.9–71.9)	n.s.	53.4 (36.9–78.2)	n.s.
Neutrophils (%)	0.5 (0.0–1.5)	0.5 (0.0–1.9)	n.s.	0.4 (0.0–1.7)	n.s.
Lymphocytes (%)	33.6 (15.5–46.7)	34.1 (7.3–19.3)	n.s.	36.1 (13.2–52.3)	n.s.
Monocytes (%)	6.7 (2.5–10.7)	6.3 (0.9–10.7)	n.s.	7.2 (5.2–12.7)	<0.05
Eosinophils (%)	2.5 (0.5–9.7)	2.5 (0.4–11.1)	n.s.	2.4 (0.5–11.8)	n.s.
Lymphocytes (abs)	2075.5 (1171.5–3136.2)	2043.4 (1320.1–2811.2)	n.s.	2032.8 (1093.0–4011.4)	n.s.
T lymphocytes (%)	71 (51–81)	70 (38–83)	n.s.	73 (48–88)	n.s.
T lymphocytes (abs)	1814 (854–2727)	1490.5 (933–2537)	<0.001	1476 (750–2531)	<0.01
CD4 (%)	44 (28–58)	43 (24–55)	n.s.	41 (29–65)	n.s.
CD4 (abs)	1122 (427–1831)	860 (504–1834)	<0.001	839 (432–1898)	<0.01
CD8 (%)	23 (10–43)	22 (13–46)	n.s.	26 (10–43)	n.s.
CD8 (abs)	539 (184–999)	489 (231–1296)	n.s.	511 (227–1156)	n.s.
B lymphocytes (%)	11 (4–24)	12 (5–20)	n.s.	11 (2–28)	n.s.
B lymphocytes (abs)	257 (89–686)	249 (115–686)	n.s.	238 (30–1124)	n.s.
Natural killer cells (%)	17 (3–45)	16 (5–45)	<0.01	14 (5–36)	n.s.
Natural killer cells (abs)	472 (80–831)	362 (105–1161)	<0.001	310 (74–703)	<0.001
CD4/CD8	1.92 (0.78–3.94)	1.93 (0.67–3.61)	n.s.	1.72 (0.72–4.57)	n.s.

Abs, absolute count; n.s., not significant or $p > 0.05$.

Table IV. Immunoglobulins and other serum immune parameters in study subjects divided according to job title.

	Exposed			Controls	
	T0	T30		T0	
	Median (min–max), *n*	Median (min–max), *n*	p⋆ for comparison exposed T0 vs. exposed T30	Median (min–max), *n*	p° for comparison exposed T0 vs. controls T0
IgA (mg ml^{-1})	2.86 (1.22–6.44), 48	4.01 (0.74–6.73), 48	<0.001	4.31 (1.36–10.90), 44	<0.001
IgG1 (mg ml^{-1})	10.30 (2.57–18.49), 48	8.8 (3.83–13.86), 48	n.s.	10.16 (4.15–16.10), 44	n.s.
IgG4 (mg ml^{-1})	0.008 (0.002–0.075), 48	0.007 (0.002–0.045), 48	n.s.	0.008 (0.0018–0.08), 44	n.s.
IgM (mg ml^{-1})	0.53 (0.32–1.14), 48	1.09 (0.24–6.36), 48	<0.001	0.90 (0.20–3.22), 44	<0.001
C3 complement (g l^{-1})	0.97 (0.64–1.44), 46	0.94 (0.63–1.38), 48	n.s.	0.97 (0.74–1.45), 45	n.s.
C4 complement (g l^{-1})	0.19 (0.13–0.40), 46	0.18 (0.12–0.35), 48	<0.001	0.19 (0.12–0.45), 45	n.s.
α1-glycoprotein (g l^{-1})	0.76 (0.50–1.46), 39	0.72 (0.45–1.31), 48	n.s.	0.72 (0.41–1.13), 35	<0.05
ESR	7 (0–21), 43	8 (1–36), 48	n.s.	9 (1–29), 40	n.s.

n.s., not significant or $p > 0.05$.

of urinary ETU to reflect only recent exposure (in the order of some days), while this biomarker cannot provide information on exposure during the whole investigated period; however, since working modalities and amounts of pesticide used in different applications were similar, we can reasonably conclude that no substantial differences in the levels of exposure of the workers under study occurred among the different treatments performed.

The significant difference in ETU excretion at T30 in vineyard workers recruited in Lombardy and in Trentino-Alto Adige is believed to reflect differences in working modalities. Particularly, in Trentino-Alto Adige workers were exposed to pesticides only during application or re-entry activities, while mixing and loading, a task known to be characterized by substantial exposure, was not performed by these subjects. Furthermore, these workers showed a better level of training, and a greater attention to health and safety practice, together with a very good level of compliance with preventive rules, among which was correct use of personal protective equipment. Despite the difference in exposure, the comparison made between immune and clinical data failed to show significant differences between these groups for any of the parameters taken into account.

During the exposure period, most of the workers were exposed to multiple active ingredients, reflecting the typical situation of agricultural workers, who often use cocktails of chemicals at the same time or different chemicals at different periods, depending on the pests to be controlled. As, apart for mancozeb, no immunotoxic activity has ever been reported for any of these compounds, and as there is no evidence of a possible interaction between the toxic mechanisms of these substances and that of mancozeb, we tend to rule out the possibility that this complex exposure could have acted as a confounder in the interpretation of our results on the effects of short-term exposure.

Exposed and controls did not significantly differ for gender, age and body mass index, but showed a significant difference for area of residence, with a higher proportion of controls living in an urban environment. This difference did not significantly affect the distribution of allergic symptoms between the study groups, although a lower incidence of allergic diseases was observed in previous studies when rural and urban populations were compared (Bibi et al. 2002, Braback et al. 2004).

In our previous investigation of the effects of mancozeb exposure on the immune status of vineyard workers we found a decrease in the percentage of monocytes (Corsini et al. 2005). In the present study we cannot confirm this observation, as in exposed subjects monocytes showed a decreasing trend from 6.7 to 6.3%, but this difference was not statistically significant. Taking into account the characteristics of working activities and occupational exposure in agriculture, we cannot rule out that the differences between the present study and the previous investigation might be due to differences in the patterns of application/exposure, for which seasonal variability is well known.

The response to vaccination, using an antigen to which no prior exposure has occurred, is considered one of the most sensitive tests for the functional assessment of the immune system in epidemiological studies (van Loveren et al. 1999, 2001). However, it is worth mentioning that the use of hepatitis B vaccination as a functional test has some practical limitations, in particular related with the fact that this practice may not be accepted by the volunteers taking part in the study, and that a significant and increasing proportion of the general population show immunizations against

hepatitis B, due either to previous contacts with the virus or, mainly, to previous vaccination, according to national rules in force in most EU countries. Also in this research only a small number of subjects could be vaccinated, and consequently the lack of difference in the levels of immunization against hepatitis B antigen between exposed subjects and controls, although consistent with the lack of relevant changes in the other immune parameters investigated, could also be due to the limited statistical power of this comparison.

Perhaps the most intriguing observation of our study is the higher proportion of exposed subjects reporting cold or flu symptoms in the 4 weeks before the investigation. This situation is also reflected by the lower plasma levels of IgA, IgM and absolute number of monocytes as well as the increased number of T lymphocytes and CD4 in exposed subjects compared with controls. In addition, we have previously demonstrated, both *in vivo* and *in vitro,* that mancozeb exposure is associated with defective cytokine production in monocytes (Corsini et al. 2006). In this light, a possible reduction in the production of tumour necrosis factor by the macrophages in the respiratory tract of the exposed subjects could result in an increased risk of infection. Yet, it remains to be established whether this finding is related to exposure to EBDCs and/or other pesticides or should be attributed to other unidentified factors, such as, for example, the difference between exposed subjects and controls in the area of residence and education (see Table I). In fact it seems unlikely that the previous year's exposure could have a carry-over effect on this symptom, while others, which may also suggest compromised immune system efficiency, would be unaffected. Moreover we observed that the altered levels of the immune parameters at T0, after the exposure period, instead of becoming more evident, returned to values similar to those found in controls. Therefore, also considering the small number of investigated subjects we suggest caution in the interpretation in these data. On the other hand, this observation is partially consistent with a previous study reporting an increase in upper respiratory tract infections in pesticide-exposed workers in comparison with the general population; however, the finding was associated with exposure to organophosphorous compounds (Hermanowicz & Kossman 1984) and not EBDCs.

In conclusion, this study suggests that the seasonal application of mancozeb, with an intermittent, but significant exposure to the fungicide, may cause slight and transient changes of some immune parameters, but does not pose a significant immunotoxic risk to exposed subjects.

On the other hand, alterations in immune status and functionality, which may be tolerated well in normal healthy adults, could have more serious consequences for those who are chronically sick, malnourished, or whose immune system has yet to develop or is in decline, underlying the importance of monitoring pesticide exposure and possible effects in the general population, with a particular attention to vulnerable subgroups.

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