Immunological changes among farmers exposed to phenoxy herbicides: preliminary observations

Annunziata Faustini, Laura Settini, Roberta Pacifici, Valeria Fano, Piergiorgio Zuccaro, Francesco Forastiere

Abstract

**Objectives**—To evaluate short term immunological changes after agricultural exposure to commercial formulations of chlorophenoxy herbicides.

**Methods**—Blood samples were collected from 10 farmers within seven days before exposure, one to 12 days after exposure, and again 50 to 70 days after exposure. Whole blood was used to count lymphocyte subsets with monoclonal antibodies. Peripheral blood mononuclear (PBM) cells were used to measure natural killer (NK) cell activity and lymphocyte response to mitogenic stimulations. Values before exposure were used as reference.

**Results**—In comparison with concentrations before exposure, a significant reduction was found one to 12 days after exposure in the following variables (P < 0.05): circulating helper (CD4) and suppressor T cells (CD8), CD8 dim, cytotoxic T lymphocytes (CTL), natural killer cells (NK), and CD8 cells expressing the surface antigens HLA-DR (CD8-DR), and lymphoproliferative response to mitogen stimulations. All immunological values found 50–70 days after exposure were comparable with concentrations before exposure, but mitogenic proliferative responses of lymphocytes were still significantly decreased.

**Conclusions**—According to our data agricultural exposure to commercial 2,4-dichlorophenoxyacetic acid (2,4-D) and 4-chloro-2-methylphenoxyacetic acid (MCPA) formulations may exert short term immunosuppressive effects. Further studies should clarify whether the immunological changes found may have health implications and can specifically contribute to cancer aetiology.

**Keywords:** immunological changes; farmers; phenoxy herbicides

Agricultural exposure to phenoxy herbicides has been associated with increased risk of non-Hodgkin’s lymphoma (NHL), and less consistently, increased risk of soft tissue sarcoma. As striking excesses of non-Hodgkin’s lymphoma have been well documented in association with immunological disorders or immunosuppressive treatment, some authors have recently suggested that phenoxy herbicides might cause this type of cancer by altering the function of lymphocytes.

A few experimental studies have been carried out in mice with purified derivatives and commercial formulations of 2,4-dichlorophenoxyacetic acid (2,4-D), an active ingredient used extensively to control broad leaf weeds. The results from these investigations showed a variety of immunological alterations: B and T lymphocyte proliferative responses were enhanced after subacute dermal exposure and acute oral exposure to derivatives of 2,4-D, whereas in utero exposure to 2,4-D ester produced a reduction of lymphocyte mitogen responses, alterations of urethane induced pulmonary adenoma were also found after exposure to a commercial formulation of 2,4-D, suggesting a mild cancer promoting activity of these products by means of alterations of cell mediated immune function.

No prospective data are available on immunological effects of phenoxy herbicides on humans. In this article we report the results of a study evaluating short term effects of agricultural exposures to phenoxy herbicides on human peripheral blood lymphocytes.

**Materials and methods**

**SUBJECTS**
The study included 10 farmers, mean (SD) age 44.0 (9.1) years, who mixed and applied chlorophenoxy herbicides during March 1994, for one to three days. All farmers used commercial formulations containing 2,4-D and 4-chloro-2-methylphenoxyacetic acid (MCPA). The mean (range) quantity of herbicides applied was 39.1 (12–155) kg.

**BLOOD SAMPLING AND IMMUNOLOGICAL ANALYSES**
Blood samples were collected and analysed within seven days before exposure to herbicides, within one to 12 days after exposure, and within 50–70 days after exposure. During the study period the farmers were not exposed to other pesticides. Blood samples were obtained in EDTA vacutainer tubes and were analysed within two hours after phlebotomy. Complete blood profile and count was obtained from each subject. Whole blood was used to count lymphocyte subsets with commercial monoclonal antibodies (CD4-FITC/HLA-DR-PE; CD8-FITC/HLA-DR-PE; CD3-PE/CD16-CD56-FITC). Stained and fixed lymphocytes were analysed with an Ortho Cytoron Absolute 4 flow cytometer.
Peripheral blood mononuclear (PBM) cells were separated from blood by density gradient centrifugation. The PBM cells were used to measure natural killer (NK) cell mediated cytotoxicity by a \(^{31}C1\)Cr release assay. The data were expressed as lytic units \(20(\text{LU}_{10})/10^7\) effector cells. To evaluate lymphocyte proliferative response, PBM cells were washed and suspended in tissue culture medium (RPMI 1640 with 10% heat inactivated fetal calf serum, L-glutamine, and penicillin/streptomycin). Cells were stimulated with different doses of phytohaemagglutinin (PHA) and concavalin A (ConA). Trinitiated methyl-\(^3H\)-thymidine (1 \(\mu\)Ci/well) was added after 24 hours of incubation. Cells were harvested on to glass fibre filters 18 hours later. Counts were expressed as counts per minute (cpm).

**STATISTICAL METHODS**

The differences between values before and after exposure were tested through the Wilcoxon test. The Spearman’s rank correlation coefficients \(r_s\) were used to test the correlation between kg of pesticides applied and the differences between values of the immunological variables before and after exposure.

**Results**

All farmers under investigation showed values before exposure for immunological variables within normal limits (table). After one to 12 days the exposure was stopped, the proportions of circulating T helper cells (CD4), suppressor T cells (CD8), CD8 dim, cytotoxic T lymphocytes (CTL), CD8 cells expressing the surface antigen HLA-DR (CD8-DR), and of NK cells were significantly reduced. Within the same period, NK cell activity and lymphoproliferative response to mitogen stimulations also were significantly reduced (table). No correlation was found between kg of pesticides applied and the decrease in the percentage of lymphocyte subsets. The correlation between decrease in NK cell activity after exposure and quantity of pesticides used was suggestive of an association, although not significantly \(r_s = 0.47, P = 0.08\). Evaluation at 50–70 days after exposure showed that the percentage of CD8-DR, NK cell activity, and mitogenic proliferative responses of lymphocytes were still significantly decreased \((P < 0.05)\), although the percentage of CD3 and CD8, which were affected immediately after exposure, recovered, showing significant increases.

**Discussion**

Experimental studies have shown that different types of pesticides are able to exert a variety of effects on the immune system in animals. Although human data on that topic are very limited, it has been suggested that immunotoxic effects of pesticides have played a part in cancers related to farming, and more specifically in the aetio-pathogenesis of B cell malignancies.

The present study is the first to show immunological changes after short term exposure to phenoxy herbicides in an agricultural setting. According to these preliminary data, commercial formulations of 2,4-D and MCPA are able to exert short lived immunosuppressive effects that decrease immediately after exposure the percentage of lymphocyte subsets CD4, CD8, CD8-DR, CD8 dim, CTL, the percentage of NK cells along with as their activity, and the lymphocyte mitogen responses.

The percentage of CD8-DR was severely reduced after exposure to phenoxy herbicides. The functional role of these cells is still unclear. It has been reported that these lymphocytes are able to inhibit cytotoxic cell activity and release a soluble lectin binding factor as the mediator of their inhibitory activity. The reduction of the percentage of CTL, NK cells, and NK cell cytotoxic activity reported here is of particular interest. These immune cells are able to recognise virally infected cells and destroy them through cytolytic mechanisms. The NK cells are also considered to be directly involved in cell mediated immunity to tumours. It has been recently suggested that other types of pesticides—that is, organophosphorous insecticides—might increase the risk of lymphomas through the impairment of cytotoxic mechanisms of immune cells.

Farmers are usually exposed to different types of pesticides, depending on the seasonal crop care in which they are involved. Both phenoxy herbicides and organophosphorous insecticides, applied during different periods of the year, could cause episodes of immunosuppression, specifically affecting NK cell mediated cytotoxicity, perhaps through different mechanisms. It may speculate that prolonged and repeated episodes of immunosuppression might be involved in the lymphomagenesis process. Interestingly, in two epidemiological studies, the risk of NHL increased according to the mean annual number of days spent mixing or applying 2,4-D.

A reduced response of lymphocytes to mitogenic stimulations was also reported in this study. A generalised suppression of the lymphocyte mitogen responses, in the absence of any structural changes in lymphoid organs, was found in mice after in utero exposure to

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**Immunological variables (means (SD)) among 10 farmers before and after exposure to phenoxy herbicides (values after exposure were compared with those before)**

<table>
<thead>
<tr>
<th>Variables</th>
<th>Before exposure</th>
<th>1–12 days after exposure</th>
<th>50–70 days after exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leucocytes ((\times 10^3))</td>
<td>6.0 (1.5)</td>
<td>6.3 (1.8)</td>
<td>5.7 (1.5)</td>
</tr>
<tr>
<td>T cells (%):</td>
<td>20 (0.3)</td>
<td>1.9 (0.5)</td>
<td>1.7 (1.5)</td>
</tr>
<tr>
<td>CD3</td>
<td>62.5 (6.9)</td>
<td>59.7 (7.4)</td>
<td>68.8 (9.3)*</td>
</tr>
<tr>
<td>CD4</td>
<td>34.0 (5.5)</td>
<td>30.0 (4.6)*</td>
<td>35.9 (5.5)</td>
</tr>
<tr>
<td>CD4-DR</td>
<td>10 (2.2)</td>
<td>8.2 (5.2)</td>
<td>10 (3.3)</td>
</tr>
<tr>
<td>CD8</td>
<td>26.0 (5.7)</td>
<td>20.2 (7.8)*</td>
<td>34.6 (8.2)*</td>
</tr>
<tr>
<td>CD8-DN</td>
<td>19 (1.70)</td>
<td>5 (2.8)*</td>
<td>7 (2.5)*</td>
</tr>
<tr>
<td>CD8 dim</td>
<td>39 (16.5)</td>
<td>28 (16.1)*</td>
<td>34 (18.9)</td>
</tr>
<tr>
<td>CTL</td>
<td>128 (4.4)</td>
<td>76 (4.0)*</td>
<td>11 (6.5)</td>
</tr>
<tr>
<td>CD4/CD8 ratio</td>
<td>1.4 (0.4)</td>
<td>1.8 (0.8)</td>
<td>1.2 (0.8)</td>
</tr>
<tr>
<td>NK cells (%)</td>
<td>15 (10.0)</td>
<td>9.8 (7.0)*</td>
<td>5.4 (2.5)</td>
</tr>
<tr>
<td>NK cell activity (LU(_{10}) × 10^3 EC)</td>
<td>214 (155)</td>
<td>100 (87)*</td>
<td>155 (82)*</td>
</tr>
</tbody>
</table>

| Lymphocyte responses to mitogenic stimulation: |
| PHA (cpm) | 85385 (10374) | 42631 (20997)* | 73942 (19856)* |
| ConA (cpm) | 40454 (10374) | 18556 (9333)* | 30746 (14359)* |

\*P < 0.05; \*P < 0.01.

LU\(_{10}\) × 10^3 EC = lytic units 20 for 10^3 effector cells; cpm = counts per minute.
pure 2,4-D esters.11 On the other hand, lymphocyte mitogen responsiveness was increased in adult mice after subacute exposure.12

Our findings are based on a longitudinal approach where immunological variables of the subjects under study before exposure were used as reference. Further studies with unexposed comparison groups are needed to confirm our preliminary data and clarify whether these types of immunological changes may have health implications, contributing specifically to cancer aetiology.

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13 Blakley BR, Blakley PM. The effect of prenatal exposure to the n-butylstearate, 2,4-dichlorophenoxyacetic acid (2,4-D) on the immune response in mice. Teratology 1986;33:15-20.