Immunotoxicity of carbaryl in chicken

B P Singh, Lokesh Singhal1 & R S Chauhan1
Department of Pathology and infectious diseases, The Royal Veterinary College, Hawkshed lane, North Mymms, Hertfordshire, AL9 7TA, England
1Centre for Animal Disease Research and Diagnosis, Indian Veterinary Research Institute, Izatnagar 243 122, India

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Effect of methyl carbonate pesticide, carbaryl, was studied on macrophage functions, lymphocyte proliferation and delayed type hypersensitivity response. Sixteen adult chicken, vaccinated against Newcastle disease, were procured and randomly divided in two experimental groups. Chicken of group I served as control, while group II birds were given carbaryl at 20 ppm (No observable effect level, NOEL) in feed for 3 months. To measure the functional activity of phagocytic cells, nitroblue tetrazolium (NBT) reduction test was performed on peripheral blood leucocytes. Concanavalin A (Con-A) and lipopolysaccharide stimulated proliferation of T and B lymphocytes was assessed using MTT dye method. At the end of experiment, the phagocytic capacities of macrophages were significantly reduced in carbaryl treated group. Lymphocyte proliferation responses to Con-A and LPS were (23 and 28%, respectively lower) in chicken fed with carbaryl. Delayed hypersensitivity reaction to tuberculin was reduced to 77% of control values indicating inhibition of cell mediated immune response. The present study suggested of immunosuppressive effect of (NOEL dose carbaryl) in chicken.

Keywords: Carbaryl, Immunotoxicity, Macrophage, Newcastle disease

Carbaryl is a wide-spectrum carbamate insecticide which controls over 100 species of insects on citrus, fruit, cotton, forests, lawns, nuts, ornamentals, shade trees, and other crops, as well as on poultry, livestock and pets. It is also used as a topical molluscicide and an acaricide1. Carbaryl works by ingestion into the stomach of the pest or absorbed through direct contact. The chemical name for carbaryl is 1-naphthol N-methyl carbamate and CAS registry no is 63-25-2(Ref.2).

Immunomodulation by agrochemicals is gaining significance in toxicity evaluation, as low level dietary intake through food residues may decrease the resistance to infectious agents and cause breakdown to vaccination3. In outdoor agricultural situation, carbaryl is rapidly destroyed by plants, bacteria, sunlight and water, but in indoor use, it can be stable for longer periods of time so that an accumulation can be anticipated. Due to its wide application, humans may be exposed to its residues through food and other routes. As it can be absorbed through the skin4, carbaryl will result in some level of exposure.

Carbaryl toxicity has been reported in human, mammals and birds5-6. It can produce adverse effects in humans by skin contact, inhalation or ingestion. Acute toxic effects of carbaryl are well documented. The immunosuppressive effect of some other carbamate pesticides like ethyl carbamate7, aminocarb8 and carbofuran9-10 have been reported. In the present investigation we have reported the immunosuppressive effects of carbaryl in chicken due to its chronic exposure.

Materials and Methods

Animals and chemical exposure regimen — Sixteen, 2-3 week old, white leghorn broiler chicken (500-600 g) were procured from Poultry Research Centre at GB Pant University of Agriculture and Technology, Pantnagar, India, and divided randomly in two equal groups. The first one received no chemical treatment and served as control, while the other group was fed with carbaryl @ 20 ppm in feed for three months. They were kept under good ventilation and hygienic conditions and allowed free access to feed and water during the study. All maintenance and care were in accordance with the animal welfare guidelines established at university. The dose of carbaryl had been selected based on its NOEL (No Observable Effect Level) value.

Blood collection/lymphocyte isolation — Blood was collected from wing vein aseptically with a 24-g needle attached with 5 ml syringe (pre-heparinized). An aliquot of this blood was placed in another tube for the whole blood and differential blood counts. Heparinized blood (3 ml) was mixed with equal
volume of RPMI-1640 medium containing 2mM of L-glutamine; 50IU/ml, penicillin; and 50mg/ml, streptomycin. Histopaque-1077 (Sigma; 3 ml) was taken in another sterile tube and media mixed blood was carefully overlaid on histopaque-1077. The tubes were then centrifuged at 400g for 15 min. The buffy coat (lymphocyte rich) was identified and was taken in another sterile tube and media mixed blood streptomycin. Histopaque-1077 (Sigma; 3 ml) was added in each well and mixed thoroughly by pipetting to solubilize the formazan crystals to produce a solution of suitable for measurement of absorbance. The results were reported as optical density (OD) measured at wavelength of 570 nm.

Delayed type hypersensitivity response — Five birds from each group were sensitized by injecting 0.25 ml of DNCB (10 mg/ml) intradermally. After two weeks of sensitization the birds were challenged with 0.25 ml of DNCB (1mg/ml) at the same area of the skin. Calliper measurements of the skin thickness were made at 0, 12, 24, 36 and 72 h following the challenge.

Statistical analysis — Student’s t test was used to estimate statistically significant differences between mean values of treated and control birds.

Results
The observations made daily during the experiment did not reveal any appreciable clinical signs of toxicity in birds of any experimental group.

Mean total leucocyte and absolute lymphocyte counts (10^7/µl) of both groups are presented in Table 1. Initially, there was no significant difference in TLC values in control and carbaryl treated group upto two months. After 2 months, mean TLC values in group II (treated group) were reduced significantly as compared to controls. There was significant suppression of mean absolute lymphocyte values in carbaryl treated group at 60, 70, 80 and 90 days (Table 1).

<table>
<thead>
<tr>
<th>Days</th>
<th>Control group</th>
<th>Carbaryl treated group</th>
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<tbody>
<tr>
<td></td>
<td>TLC</td>
<td>ALC</td>
</tr>
<tr>
<td>10</td>
<td>16.35±0.23</td>
<td>12.49±0.17</td>
</tr>
<tr>
<td>20</td>
<td>18.32±0.12</td>
<td>14.25±0.29</td>
</tr>
<tr>
<td>30</td>
<td>20.28±0.49</td>
<td>15.83±0.38</td>
</tr>
<tr>
<td>40</td>
<td>23.39±0.36</td>
<td>18.36±0.28</td>
</tr>
<tr>
<td>50</td>
<td>24.28±0.63</td>
<td>19.10±0.49</td>
</tr>
<tr>
<td>60</td>
<td>27.77±0.79</td>
<td>21.93±0.62</td>
</tr>
<tr>
<td>70</td>
<td>29.12±0.73</td>
<td>23.09±0.57</td>
</tr>
<tr>
<td>80</td>
<td>30.18±0.35</td>
<td>24.70±0.28</td>
</tr>
<tr>
<td>90</td>
<td>32.38±0.32</td>
<td>24.96±0.28</td>
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</tbody>
</table>

Table 1 — Effect of carbaryl on total leucocyte counts (TLC) and absolute lymphocyte counts (ALC) of blood in chicken

| Values are mean ± SE of 4 replications |

*Significant at P≤0.5

TLC and ALC have been represented as 10^7/µl.
the end of second month in comparison to control. At
the end of experiment, ALC value in treated group was
21.38±0.32 as compare to 24.96±0.28 in controls.

Nitro blue tetrazolium exposed cells, which were able
to convert the yellow coloured dye into bluish dark
granules and become recognizable, were designated as
NBT positive cells. These cells were expressed as per
cent and the data are presented in Fig. 1. The mean per
cent NBT positive cells in carbaryl treated group
(22.5±1.28) were significantly less as compare to
controls (33.7±1.43) after 2 months of treatment. At the
end of third month there was about 39 per cent reduction
in NBT positive cells in carbaryl fed birds (21.1±1.62) in
comparison to controls (34.3±1.12).

Mean delta OD of control and carbaryl fed group
has been shown in Fig. 2. A significant reduction in
mean delta OD of treated birds was observed for B and T lymphocytes from 60th day post treatment till the end of the experiment, which indicated decrease in lymphocyte proliferative response for mitogens in treated group. After three months of experiment, mean delta OD in carbaryl treated birds were reduced 28 and 23 per cent for B and T lymphocyte proliferation, respectively.

Results of DTH reaction are presented in Table 2. Peak reaction was observed at 24 h followed by subsequent decrease by 48 h and negligible reaction at 72 h post challenge with DNCB. Histopathological examination of section of tissues at different intervals was carried out to evaluate various possible pathomorphological changes. No alteration could be detected in both groups at 0-4 h of post inoculation. At 24 h, extensive infiltration of mononuclear cells and few neutrophils were observed in both groups. The reaction was less pronounced in carbaryl treated group as compared to control group. A direct correlation between increased thickness and extent of infiltration of mononuclear cells was noticed.

Discussion
The present work focused on the effect of carbaryl on the immune system of broiler chicken. To understand the toxic effects and mechanism of action of pesticide is quite essential to assess the public and environmental health risks. Many assays have been developed to look the acute toxicity, mutagenic, carcinogenic and treatogenic effects of pesticide. Less attention has been paid to evaluate the effect of pesticide on the immune system. The broiler chicken were used as a model in our study as the immune system of chicken has been well studied and is quantifiable by several techniques. Evaluation of immune functions of chicken is considered a sensitive indicator of immunotoxic effect of the contaminants. The sensitivity of chicken to toxic effect of xenobiotics has served to alert the scientific and regulatory communities to the presence of such contaminants before wide spread human exposure occurred.

Suppression of TLC and ALC has been observed earlier in sheep fed with another carbamate pesticide carbofuran for six months. The leucopenia observed in the present study may be due to cytotoxic effects of carbaryl. Since the lymphocytes are the main cells to play vital role in defence mechanism, reduction in number of absolute lymphocytes as revealed in the present study is an indication of immunosuppression.

Decrease in NBT positive cells indicates the reduced functional status of phagocytic cells. Phagocytic cells are considered as important component of defense mechanisms as they act against any foreign invasion not only to kill and remove them from the body but also these cells act as antigen presenting cells and participate actively in the specific immunity. The reduction in number of active phagocytic cells in carbaryl treated birds may also lead to decreased natural resistance or innate immunity to infections. Proliferative assay of lymphocytes to ConA and LPS mitogens have been suggested as a measurement of lymphocyte proliferation capacity. Mitogens stimulate the proliferation of lymphocytes independent of their antigen specificity. This proliferative response is considered to reflect clonal expansion of that follows antigen sensitization "in vivo". Lipopolysaccharide (LPS) and concanavalin (Con-A) stimulate B and T cells, respectively. Lymphocyte proliferation assay using MTT is a rapid calorimetric assay and has number of advantages over the conventional assays. This test is based on the capacity of mitochondrial enzymes succinate dehydrogenase to transform the tetrazolium salt of MTT in to a blue colour product, formazon that can be quantified spectrophotometrically. Significant depression in proliferation of B-lymphocytes indicates the lowered capacity of B-lymphocytes to form clones and convert into

<table>
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<th>Group</th>
<th>0</th>
<th>12</th>
<th>24</th>
<th>36</th>
<th>72</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.83±0.14</td>
<td>1.97±0.2</td>
<td>2.56±0.13</td>
<td>2.28±0.14</td>
<td>1.85±0.13</td>
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<tr>
<td>Carbaryl</td>
<td>1.81±0.12</td>
<td>1.93±0.14</td>
<td>1.98±0.24*</td>
<td>1.64±0.16*</td>
<td>1.74±0.17</td>
</tr>
</tbody>
</table>

*Significant at P<0.5

Table 2— Effect of Carbaryl on delayed type hypersensitivity (DTH) reaction. Mean skin thickness, mm ± S.E.M.

[Values are mean ± SE of 4 replications]
plasma cells. Plasma cells are responsible for the secretion of antibodies and thus it indicates that B-lymphocytes have become less responsive to antigen leading to suppression in humoral response. Impaired humoral response may enhance the chances of vaccinal failures and susceptibility to infections.

Carbaryl lowered the cell mediated immune response as shown by significant reduction in DTH reaction to DNCB. These findings indicated functional impairment of another subpopulation of T- cells (T-affector cells)21. Following interaction with a specific antigen, these cells are responsible for secretion of lymphokines. In DTH reaction, the primary lymphocytes response involved appears to be responsible for accumulation of mononuclear cell infiltrate, their interaction and increased vascular permeability occurs in the vicinity of the stimulus22. This finding further gets support as we reported decreased number of total leucocyte and absolute lymphocyte counts in carbaryl treated group.

Immunotoxic influence of carbaryl is in agreement with previous studies concerned with other carbamate pesticides. Carbaryl has been shown to inhibit humoral immunity in mice23,24 and lowered resistance of quails8. In vitro, carbaryl impaired the immune function of splenocytes3 and inhibited proliferation of IL-2 dependent T cells25. Carbofuran suppresses both humoral8,10 and cellular immunity9 and inhibits the expression of complement activity when added to human serum26. Aminocarb reduces humoral immunity and increases cytolysis of macrophages by virus8,27. Another carbamate pesticide, previcur, has been studied for its immunotoxicological properties and found to suppress the humoral and cell mediated immunity in mice28. Carbendazim at NOEL dose also down regulates the humoral immunity in chicken29. Chronic exposure of aldicarb or ethyl carbamate lowered humoral immune response in mice30-32. In contrast, of some reports9,33 carbaryl or aldicarb did not affect humoral and cellular immune responses.

Cholinesterase depression is common to all the carbamate pesticide both in blood and tissues. Inhibition of cholinesterase cause accumulation of acetylcholine in synapses, resulting in different malfunctions of the nervous system34. There is little information concerned with the mode of action of carbamate pesticides on immunity. It has been reported3 that carbamates act through inhibition of serine esterase to modulate the generation and expression of immune response. The immunosuppression may result from direct action of acetylcholine upon the immune system or it may be secondary to the toxic chemical stress associated with cholinergic poisoning35. Aldicarb has no direct effect on T cells and the mechanism of modulation indirectly mediated through impairment of macrophage ability to secrete interleukin-1 (IL-1), which is critical for T cell activation. In addition, the decrease in IL-1 production may also affect the production of other cytokines including IL-2, IL-4, IL-6 and IL-822,35-36. This will in turn affect the regulation of the interaction and function of antigen-presenting cells, T helper cells and B cells37.

Depressed immune response may be related to inhibition of energy metabolism. Impaired energy production may result from inhibition of the activity of mitochondrial pyruvate and $\alpha$-ketoglutarate dehydrogenase complex. Energy is required for biosynthesis of numerous molecules that participate in many biochemical reactions in the immune system. Therefore, reduced ATP formation is likely to affect synthesis of cellular components of the immune system. Suppression in humoral and cell mediated immunity have been documented in chicken with energy deficiency38,39.

In conclusion, dietary exposure of carbaryl in chicken for three months at 20 ppm significantly reduced the macrophage phagocytic capacity, Con A and LPS induced proliferation capacity of T and B lymphocytes and DNCB induced delayed hypersensitivity response.

Acknowledgement

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