EFFECTS OF NOCARDIA RUBRA CELL WALL SKELETON ON INTERLEUKIN 2 PRODUCTION AND LYMPHOCYTE PROLIFERATION IN FORMER POISON GAS FACTORY WORKERS

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Phytohemagglutinin (PHA)-induced interleukin 2 (IL-2) production and lymphocyte proliferation were measured in former workers of the Okunojima Poison Gas Factory (poison gas workers), who have a high incidence of lung cancer, and the efficacy of administration of Nocardia rubra cell wall skeleton (N-CWS) was studied. In comparison with normal controls and poison gas workers receiving N-CWS, lymphocyte proliferation in poison gas workers not receiving N-CWS showed a significant decrease, while IL-2 production showed a slight though not statistically significant decrease. When N-CWS was administered to poison gas workers, IL-2 production and lymphocyte proliferation were significantly elevated, with a peak two weeks after administration. N-CWS, by elevating IL-2 production of lymphocytes, is considered to have improved the depression of lymphocyte proliferation.

Key words: Interleukin 2 production — Lymphocyte proliferation — Poison gas worker — Nocardia rubra cell wall skeleton

In 1977, interleukin 2 (IL-2)-dependent mouse cytotoxic T cell line (CTLL-2)1 was established by Gillis et al., and with the establishment of a microassay using CTLL-2,2 IL-2 activity in culture supernatant can be measured with ease. This IL-2 assay has also been utilized for the purpose of elucidating the behavior of IL-2 in diseases with immune abnormalities. A number of papers have already been published reporting that IL-2 productivity of lymphocytes in animals and man is depressed in systemic lupus erythematosus (SLE) and in malignant diseases.3-5

In order to examine the possibility of cancer prevention by immunopotentiation in the former workers of the Okunojima Poison Gas Factory (poison gas workers), who have a high incidence of lung cancer6 and depressed immunity, we administered intra-dermally 200 µg of Nocardia rubra cell wall skeleton (N-CWS) in about 200 poison gas workers every three months and followed the changes in immunological parameters. The present paper reports on phytohemagglutinin (PHA)-induced IL-2 production and lymphocyte proliferation in poison gas workers and on the effect of N-CWS administration on these parameters.

MATERIALS AND METHODS

The subjects of the present study were 172 males composed of 44 normal aged subjects, 69 poison gas workers not receiving N-CWS, and 59 poison gas workers receiving N-CWS once every three months; background factors are given in Table I. Normal aged subjects were aged volunteers with no clinically apparent disease, whose mean age was similar to those of poison gas workers not receiving N-CWS and all the poison gas workers receiving N-CWS. All the poison gas workers not receiving N-CWS and all the poison gas workers receiving N-CWS were suffering from chronic bronchitis which satisfied the definition given by Fletcher,7 and their mean estimated duration of chronic bronchitis is given in Table I. None of these workers had been administered steroids, antibiotics, or anti-inflammatory agents nor had they received X-ray examinations within the previous month, and there was no evidence of acute aggravation of chronic bronchitis during the previous three months. As
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shown in Table I, no remarkable difference in smoking history was observed among the three groups. Among poison gas workers, the longer the time the type of work required the worker to be in contact with mustard gas in the poison gas factory and the longer the duration of work in which the worker was engaged, the higher was the incidence of malignant tumors.8,9) The type of work was classified according to the period of contact with mustard gas into groups, A, B, and C. Workers who were directly engaged in production of mustard gas were classified into group A; workers who were engaged in laboratory, repair, and incineration work and who had many opportunities of contacting mustard gas (though less than group A) were classified into group B; and workers who had little opportunity to be in contact with mustard gas, that is, those who were engaged in the production of gases other than mustard gas (sneezing gas, tear gas and others), medical doctors, and those engaged in clerical work were classified into group C. Furthermore, the duration of work was classified into three groups, that is, less than 2 years, 2 to 5 years, and more than 5 years. For the purpose of studying the effect of type of work and duration of work on IL-2 production and lymphocyte proliferation, the subjects of the present study were chosen so that the number of poison gas workers not receiving N-CWS and the number of poison gas workers receiving N-CWS would be almost the same in number by type of work and duration of work. The detailed breakdown is shown in Table I. A dose of 200 μg of N-CWS has been given subcutaneously once every three months to approximately 200 volunteers among the poison gas workers. In the present study, 59 poison gas workers receiving N-CWS were employed, whose periods of administration of N-CWS were distributed from 24 to 60 months (50.5±9.5 months on average). From these subjects, heparinized blood was drawn at random for the determination of IL-2 production and lymphocyte proliferation. In the poison gas workers receiving N-CWS, the period from the last administration of N-CWS to the blood drawing was distributed at random from 0 to 12 weeks (5.85±3.60 weeks on the average). For the 15 cases given an initial administration of N-CWS, the changes over time of lymphocyte responses were observed immediately prior to, and 1, 2, 4 and 12 weeks after one administration of 200 μg of N-CWS.

Table I. Background Factors of the Subjects

<table>
<thead>
<tr>
<th></th>
<th>Normal aged subjects</th>
<th>Poison gas workers not receiving N-CWS</th>
<th>Poison gas workers receiving N-CWS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of cases</td>
<td>44</td>
<td>69</td>
<td>59</td>
</tr>
<tr>
<td>Age (years old)</td>
<td>68.9±8.0</td>
<td>68.0±6.5</td>
<td>69.6±7.4</td>
</tr>
<tr>
<td>Sex</td>
<td>all male</td>
<td>all male</td>
<td>all male</td>
</tr>
<tr>
<td>Clinical state</td>
<td>no clinical abnormality</td>
<td>all chronic bronchitis</td>
<td>all chronic bronchitis</td>
</tr>
<tr>
<td>Smoking</td>
<td>smoker</td>
<td>27 (61.4%)</td>
<td>39 (56.5%)</td>
</tr>
<tr>
<td></td>
<td>(696±402)</td>
<td>(651±243)</td>
<td>(669±249)</td>
</tr>
<tr>
<td></td>
<td>ex-smoker</td>
<td>6 (13.6%)</td>
<td>12 (17.4%)</td>
</tr>
<tr>
<td></td>
<td>(608±486)</td>
<td>(598±391)</td>
<td>(603±394)</td>
</tr>
<tr>
<td></td>
<td>non-smoker</td>
<td>11 (25.0%)</td>
<td>18 (26.1%)</td>
</tr>
<tr>
<td>Type of work&lt;sup&gt;a)&lt;/sup&gt;</td>
<td>group A</td>
<td>25 (36.2%)</td>
<td>20 (33.9%)</td>
</tr>
<tr>
<td></td>
<td>group B</td>
<td>22 (31.9%)</td>
<td>19 (32.2%)</td>
</tr>
<tr>
<td></td>
<td>group C</td>
<td>22 (31.9%)</td>
<td>20 (33.9%)</td>
</tr>
<tr>
<td>Duration of work&lt;sup&gt;b)&lt;/sup&gt;</td>
<td>2 years</td>
<td>24 (34.8%)</td>
<td>18 (30.5%)</td>
</tr>
<tr>
<td></td>
<td>2-5 years</td>
<td>27 (39.1%)</td>
<td>20 (33.9%)</td>
</tr>
<tr>
<td></td>
<td>5- years</td>
<td>18 (26.1%)</td>
<td>21 (35.6%)</td>
</tr>
<tr>
<td>Duration of chronic bronchitis (years)</td>
<td>19.3±12.0</td>
<td>20.3±10.2</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>) Mean value of Brinkman's index.
<sup>b</sup>) Numbers of poison gas workers by type of work in the poison gas factory.
<sup>c</sup>) Numbers of poison gas workers by duration of work in the poison gas factory.
Peripheral blood lymphocytes (PBL) separated from heparinized blood by the Ficoll-Hypaque density gradient centrifugation method were suspended in RPMI 1640 medium (Gibco) supplemented with 2.5% heat-inactivated fresh human AB serum and 1% PHA-M (Difco) to make 1 x 10⁶ cells/ml, and cultured for 24 hr at 37° under 5% CO₂. The culture supernatant was collected and cryopreserved at -80°.

**IL-2 Assay**

IL-2 activity was determined according to the method of Gillis et al. Briefly, with the use of Cline's medium supplemented with 2% heat-inactivated fetal bovine serum (Flow Laboratories), serial log₂ dilutions in eight stages were added at 100 µl/well to the 96-well microplate. To each well of this microplate, CTLL-2 cells were added to make 5 x 10⁵ cells/well, followed by incubation for 24 hr at 37° in 5% CO₂. Four hours prior to completion of incubation, 0.4 µCi of ³H-thymidine (³H-TdR) was added to each well and the radioactivity incorporated into CTLL-2 cells was measured.

**Determination of Units of IL-2 Activity** (Fig. 1) An IL-2 sample of 50% titer obtained in the same manner as described above from one human was employed as standard IL-2 (1 unit). Units of IL-2 activity were obtained by probit analysis. The maximum value of ³H-TdR incorporation (counts per minute, cpm) in CTLL cells on serial log₂ dilution of standard IL-2 (Fig. 1A, a) was designated as 100%, and each incorporation, converted into %, is plotted on the probit axis (B). The unit of IL-2 activity of a sample is computed by using the following equation. Unit = IL-2 sample dilution multiple at 50% activity of standard IL-2 (b)/standard IL-2 dilution multiple at 50% activity of standard IL-2 (c).

**Lymphocyte Proliferation Assay** In a 96-well microplate, 1.25 x 10⁵ cells/well of PBL were added, and after incubation for 96 hr in RPMI 1640 medium supplemented with 1% PHA (Welcome) and 10% heat-inactivated fresh human AB serum, the incorporation of radioactivity in PBL was measured as cpm.

The culture conditions employed for IL-2 production and lymphocyte proliferation were those described previously.

In the present study, the same two controls (a 29-year-old male and a 21-year-old female) were used on each day of the experiment and it was confirmed that there was no marked inter-day variation (data not shown). Furthermore, of the 15 cases given a single administration of N-CWS
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and followed for changes of parameters over time, an identical measurement was conducted 12 weeks and 24 weeks prior to N-CWS administration on 9 or 10 cases to confirm that there were no remarkable variations.

Student's t-test was employed to test the significance of differences. All data are expressed as average and standard deviation.

RESULTS

IL-2 Production and Lymphocyte Proliferation in Normal Controls, Poison Gas Workers not Receiving N-CWS and Poison Gas Workers Receiving N-CWS (Table II) Poison gas workers not receiving N-CWS presented in comparison with normal controls significantly low lymphocyte proliferation \( (P<0.001) \), whereas IL-2 production only showed a slightly low value with no significant difference from the controls. In comparison with poison gas workers not receiving N-CWS, lymphocyte proliferation in poison gas workers receiving N-CWS showed a significantly high value \( (P<0.001) \), but IL-2 production presented only a slightly elevated value without any significant difference. The effect of type of work and duration of work was examined, but no significant difference could be demonstrated in any category (data not shown).

Changes of IL-2 Production and Lymphocyte Proliferation in Poison Gas Workers after Administration of N-CWS IL-2 production and lymphocyte proliferation immediately prior to N-CWS administration did not show any significant inter-day variation in comparison with those 12 weeks and 24 weeks prior to N-CWS administration (Table III).

Following administration of N-CWS to 15 poison gas workers, a significant elevation of IL-2 production was observed 1 week \( (P<0.05) \) and 2 weeks \( (P<0.01) \) after administration and significantly elevated lymphocyte proliferation 2 weeks \( (P<0.05) \) and 4 weeks \( (P<0.05) \) after administration (Figs. 2 and 3).

Table II. PHA-induced IL-2 Production and Proliferation of Lymphocytes in the Poison Gas Workers

<table>
<thead>
<tr>
<th>No. of cases</th>
<th>PHA-induced response of lymphocytes</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IL-2 production (units)</td>
<td>Proliferation (cpm)</td>
</tr>
<tr>
<td>Normal aged subjects</td>
<td>44</td>
<td>2.76±1.38</td>
</tr>
<tr>
<td>Poison gas workers not receiving N-CWS</td>
<td>69</td>
<td>2.58±1.05</td>
</tr>
<tr>
<td>Poison gas workers receiving N-CWS</td>
<td>59</td>
<td>2.74±1.24</td>
</tr>
<tr>
<td>(^{a}) Statistical significance was noted as compared with the level of poison gas workers not receiving N-CWS: ( P&lt;0.001 ).</td>
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</tbody>
</table>

Table III. Changes of PHA-induced IL-2 Production and Proliferation of Lymphocytes in the Poison Gas Workers after Administration of N-CWS

<table>
<thead>
<tr>
<th>Duration after N-CWS injection (weeks)</th>
<th>No. of cases</th>
<th>PHA-induced response of lymphocytes</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>-24</td>
<td>9</td>
<td>2.49±0.52</td>
<td>21,238±6,121</td>
</tr>
<tr>
<td>-12</td>
<td>10</td>
<td>2.49±0.46</td>
<td>22,284±7,598</td>
</tr>
<tr>
<td>0</td>
<td>15</td>
<td>2.59±0.47</td>
<td>24,515±8,525</td>
</tr>
<tr>
<td>(N-CWS injection)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>12</td>
<td>3.15±0.83 (^{a})</td>
<td>31,047±8,889</td>
</tr>
<tr>
<td>2</td>
<td>14</td>
<td>3.38±0.72 (^{b})</td>
<td>32,997±9,041 (^{a})</td>
</tr>
<tr>
<td>4</td>
<td>14</td>
<td>2.68±0.43</td>
<td>31,902±7,143 (^{a})</td>
</tr>
<tr>
<td>12</td>
<td>14</td>
<td>2.48±0.47</td>
<td>28,614±6,946</td>
</tr>
<tr>
<td>(^{a}) Statistical significance as compared with the level at 0 week: ( a) P&lt;0.05 ) ( b) P&lt;0.01 ).</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Fig. 2. Changes of PHA-induced IL-2 production of lymphocytes in 15 poison gas workers before and after administration of N-CWS. Results are expressed as the mean±SD.

Fig. 3. Changes of PHA-induced proliferation of lymphocytes in 15 poison gas workers before and after administration of N-CWS. Results are expressed as the mean±SD.
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DISCUSSION

Various types of poison gases were produced at the former Okunojima Poison Gas Factory. It has been reported that the greater the frequency of exposure of workers to these poison gases, mustard gas in particular, the higher was the incidence of malignant diseases including respiratory neoplasms.6,8) The carcinogenicity of mustard gas has been demonstrated in animal experiments,11) and in man it has been reported that the incidence of respiratory tract cancers was high in servicemen of the Allied Forces exposed to mustard gas during the First World War.12) With the aim of studying the association between carcinogenesis and immunity, various immunological parameters of the poison gas workers have been measured. We have reported that among these poison gas workers there are cases whose NK cell activity was remarkably depressed,13) and that in the poison gas workers abnormalities of the peripheral blood lymphocyte subsets were observed, i.e., increase of Leu-2a+ cells, accompanied by depression of Leu-3a/Leu-2a ratio and decrease of Leu-7+ cells.14) In the present work, PHA-induced lymphocyte proliferation of the poison gas workers was significantly depressed when compared with normal controls, while IL-2 production of the poison gas workers was only slightly depressed (not statistically significant) when compared with normal controls.

N-CWS is an immune adjuvant developed by Yamamura et al. and its inhibitory effect on tumor development in mice15) and rats16) has been widely studied. Its efficacy against lung cancers17) and malignant pleurisy18) in man has been reported. Furthermore, preventive effects of N-CWS on chemically induced lung cancer in rabbits19) and rats20) and on spontaneously developing breast cancer in mice21) have been reported. On the other hand, with regard to the mechanism of the biological activity of N-CWS, it has been reported that N-CWS enhances the cytotoxicity of effector cells such as cytotoxic T cells,22) natural killer and killer cells,13) and macrophages.23) We found that both PHA-induced IL-2 production and lymphocyte proliferation were enhanced by single administration of N-CWS to poison gas workers. Furthermore, on repeated administration of N-CWS every three months to poison gas workers, lymphocyte proliferation was found to be elevated, while IL-2 production was not.

With regard to the association between IL-2 production and lymphocyte proliferation, we found a significant positive correlation between these two parameters in normal controls, poison gas workers not receiving N-CWS, and poison gas workers receiving N-CWS (P<0.001 in all groups, data not shown). As described in this report, PHA-induced lymphocyte proliferation is depressed in poison gas workers and N-CWS, by enhancing IL-2 production, is considered to correct this situation.

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REFERENCES


