Effects of periodic administration of *Nocardia rubra* cell-wall skeleton on immunoglobulin production and B-cell-stimulatory factor activity in vitro in workers at a poison gas factory

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Summary. The former workers at the Okunojima poison gas factory (poison gas workers) are a high-risk group for malignant neoplasms and show abnormalities in cellular immunity. At the same time, poison gas workers often have chronic respiratory diseases, such as chronic bronchitis, and are highly susceptible to respiratory infections. To explore the possibility of immunological cancer prevention, we have periodically administered 200 μg *Nocardi*a rubra cell-wall skeleton (N-CWS) to poison gas workers every 3 months since December 1978. During this period, we noted a significantly lower incidence of influenza among poison gas workers receiving N-CWS than in those not receiving the drug during the influenza epidemic. This finding suggested that the administration of N-CWS enhanced the resistance of these workers to infections. Therefore, periodical administration of N-CWS to poison gas workers was considered to enhance the reduced T-cell function of normalizing antibody production by stimulating the production of B-cell-stimulatory factor (BSF). In the present study, to clarify the mechanism of immunosuppression in the poison gas workers and to examine the effects of continual administration of N-CWS on this condition, we compared the immunoglobulin production and the proliferative and differentiative activities of B-cell-stimulatory factor (BSF) of peripheral blood mononuclear cells (PBMC), in poison gas workers treated or not treated with N-CWS. Comparisons were also made with age-matched healthy controls. In the untreated poison gas workers, immunoglobulin and BSF production of PBMC were reduced as compared with the control group. On the other hand, in the poison gas workers receiving N-CWS, immunoglobulin and BSF production of PBMC were restored nearly to the control level. These results show that in vitro antibody production in the poison gas workers was reduced and that a reduction in BSF production of T cells was one of its causes.

Introduction

The Okunojima poison gas factory was established by the former Japanese army in 1929 on a small island called Okunojima in the Seto Inland Sea. Until 1945, mostly mustard gas was made there [21, 22].

Through our clinical observation of men retired from the Okunojima poison gas factory (poison gas workers) after more than 30 years of work, it has been reported that they are a high-risk group for cancer, notably lung cancer [16, 17, 21, 22], and show abnormalities in the cell-mediated immune functions, such as natural killer cell activity, responsiveness to mitogens, and macrophage function [23, 25–28]. This impairment of cellular immunity has been regarded as a background for the high incidence of cancers, which are more notable in those who are considered to have been exposed more extensively to mustard gas [16, 17]. At the same time, poison gas workers often have chronic respiratory diseases, such as chronic bronchitis, and are highly susceptible to respiratory infections [15]. Impairment of cellular immunity appears to be an important factor in the reduced resistance to infections in these individuals.

To explore the possibility of immunological cancer prevention, we have periodically administered *Nocardia rubra* cell wall skeleton (N-CWS), developed by Yamamura and Azuma et al. [1–3], to poison gas workers since December 1978. During this period, Yamakido et al. noted a significantly lower incidence of influenza among those receiving N-CWS than among those not receiving the drug during the influenza epidemic in 1982 [24]. This clinical finding suggested that the administration of N-CWS enhanced resistance of these workers to infections. Yamakido et al. further reported increases in the natural killer cell activity, interferon activity, responsiveness to phytohemagglutinin and concanavalin A, and interleukin-2 production, i.e. enhancements in T cell functions, in poison gas workers treated with N-CWS [23, 26], and considered that the increased resistance to infections in these workers might be based on activation of immunocompetent cells by the agent.

Recently, cytokines derived from T cells and macrophages have been shown to be closely involved in B-cell proliferation and differentiation into antibody-producing cells [6, 7, 13, 14]. Therefore, in the poison gas workers, who are known to have various impairments of cellular immunity, especially in T cell function [23, 25–28], antibody production is likely to be reduced, but the administration of N-CWS is considered to reverse this reduction and induce enhanced resistance to infection. To clarify the mechanism of immunosuppression in the poison gas wor-
Materials and methods

Subjects. The study included 47 healthy elderly individuals (control group), 73 poison gas workers not treated with N-CWS (untreated group), and 59 poison gas workers treated with N-CWS (treated group). The clinical profiles of the subjects are summarized in Table 1. The control group consisted of men aged 50 years or above (mean 70.1 ± 7.8 years) with no apparent clinical diseases, who were not poison gas workers. The untreated group consisted of male poison gas workers with a mean age of 70.5 ± 6.6 years, all having chronic bronchitis. The treated group consisted of male poison gas workers, also with chronic bronchitis, aged 70.5 ± 6.6 years, who had received a subcutaneous injection of 200 μg N-CWS in both upper arms once every 3 months, to a total of 14-27 injections (mean, 23.0 ± 3.3 injections). Brinkman's index: 577.1 ± 509.0 for the control group, 626.3 ± 456.2 for the untreated group, and 619.5 ± 403.9 for the treated group, showed no significant differences among the three groups. In the treated group, the blood was drawn 1 month or more after the last injection.

Cell separations. PBMC were separated from heparinized peripheral blood by Ficoll-Hypaque density gradient centrifugation. The B-cell fraction was obtained by depletion of T cells and adherent cells from PBMC as described below. T-cell depletion was accomplished by treating PBMC twice by the E-rosette formation procedure, using sheep red blood cells treated with 2-aminoethylisothiouronium bromide hydrobromide. To remove the residual T cells, this was followed by complement-dependent cytolysis using anti-Leu 1 antibody (Becton-Dickinson) and rabbit complement (Pel Freez). Depletion of adherent cells was accomplished by adhering T-cell-depleted cells to a plastic flask at 37°C for 2 h. Removal of T cells and adherent cells yielded a B-cell fraction, in which the percentage of surface-immunoglobulin-positive cells was 70%-85%, 1% or less were positive for E-rosette formation (using the above procedure), 1% or less were positive for Leu-1 assayed by the indirect immunofluorescence technique and 5% or less were positive for non-specific esterase.

PWM-induced immunoglobulin production. PBMC were washed at least five times, seeded in a 96-well flat-bottomed microtest plate at 2.5 x 10^6 cells/ml, and cultured in RPMI 1640 medium containing 0.5% PWM (Gibco) and 10% heat-inactivated fetal bovine serum at 37°C in 5% CO2 for 7 days. The IgG, IgM, and IgA levels in the culture supernatant were determined by enzyme-linked immunosorbent assay (ELISA).

BSF production. PBMC were suspended at 1 x 10^6 cells/ml in RPMI 1640 medium containing 2 μg/ml phytohemagglutinin (Wellcome) and 2.5% heat-inactivated fetal bovine serum and cultured at 37°C in 5% CO2 for 72 h. Phytohemagglutinin blasts were suspended in the culture supernatant at 1 x 10^6 cells/ml, and incubated overnight at 4°C. The culture supernatant was dialyzed, sterilized by passing it through a 0.22 μm filter, and used as the BSF sample.

Measurement of proliferative activity of BSF. The proliferative activity of BSF was evaluated according to the reports of Muraguchi and Fauci et al. [10-12]. Briefly, using RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, serial log2 dilutions of the BSF sample in six stages were added at 0.1 ml/well to the 96-well microtest plate. B cells isolated as above were suspended in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum at 2 x 10^6 cells/ml, and 0.1 ml suspension was seeded in the wells of the microplate. Goat anti-human IgM [F(ab')2 fragment], μ-chain-specific (Tago), was added to the wells to a final concentration of 10 μg/ml, and the cells were incubated at 37°C in 5% CO2 for 72 h. [3H]-Thymidine (1 μCi) was added to each well 16 h before the end of culture, and the [3H]T incorporation by B cells was examined. As described by Gillis et al. [4], the [3H]T incorporation by B cells, at serial log2 dilutions of BSF sample in six stages, was converted by probit analysis to appropriate units, using human B-cell growth factor (BCGF, CPI) with 25% potency as a standard (1 unit).

Measurement of differentiative activity of BSF. The differentiative activity of BSF was evaluated according to the IgG production induced in B cells in the presence of the BSF sample [8]. B cells, isolated as above, were seeded in a 96-well microtest plate at a final concentration of 2 x 10^6 cells/ml in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum. The BSF sample was added to each well at a final concentration of 25%, and goat anti-human IgM was then added at a final concentration of 10 μg/ml. After culturing at 37°C in 5% CO2 for 7 days, the IgG concentration in the culture supernatant was determined by ELISA. The differentiative activity in the BSF was expressed as the IgG level in the culture supernatant.

PWP induction of immunoglobulin production. The proliferative and differentiative activities of BSF were determined in triplicate, and expressed as the mean of the three determinations. A sample obtained from a healthy subject (28-year-old man) was evaluated as the control on

Table 1. Background of the subjects

<table>
<thead>
<tr>
<th>Factor</th>
<th>Normal-aged control</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>47</td>
<td>73</td>
<td>59</td>
</tr>
<tr>
<td>Age (years)</td>
<td>70.1 ± 7.8^b</td>
<td>70.5 ± 7.3^b</td>
<td>70.5 ± 6.6^b</td>
</tr>
<tr>
<td>Sex</td>
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<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 status</td>
<td>577.1 ± 509.0^b</td>
<td>626.3 ± 456.2^b</td>
<td>619.5 ± 403.9^b</td>
</tr>
</tbody>
</table>

^a N-CWS, Nocardia cell-wall skeleton
^b Values are means ± SD