

Immunoregulatory T-Lymphocyte Functions in Patients with Small Cell Lung Cancer¹

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ABSTRACT

The present study was performed to elucidate the differences in immune status between patients with small cell lung cancer (SCLC) and those with non-small cell lung cancer. The study group consisted of 18 patients with SCLC and 15 with non-SCLC. Two healthy volunteers and 13 patients with benign disease were also included in the present study as the non-cancer control. In the non-SCLC group, although not statistically significant, the percentages of both OKT3+ and OKT4+ T-lymphocytes in the peripheral blood lymphocytes (PBL) were slightly decreased, associated with a slight increase in the percentage of OKT8+ T-cells, and a slight decrease in the OKT4+ to OKT8+ T-cell ratio. In contrast, the PBL of the SCLC patients showed significantly lower proliferative responses to phytohemagglutinin and human recombinant interleukin 2 than did the PBL of both the SCLC patients and the non-cancer control group. The ability of PBL to produce lymphokines (interleukin 2 and macrophage activating factor) was significantly impaired in the SCLC group but not in the non-SCLC group. These results suggest that suppression of helper T-cell functions and/or potentiation of suppressor T-cell functions should occur in patients with SCLC.

INTRODUCTION

It has been well documented that conditions associated with immune depression, whether genetic, induced by drugs, or of other origin, increase the likelihood of malignant neoplasms. On the other hand, evidence has mounted in recent years that patients with malignant tumors have a defective immune status (1-3). The host immune response to malignant disease appears to be a significant factor in determining the prognosis (4). SCLC³ has a worse prognosis than non-small cell lung cancer (5) since SCLC grows rapidly and has a high metastatic potential. Together with these findings, it can be speculated that immunocompetence is more profoundly suppressed in SCLC patients than in non-SCLC patients; however, there have been few investigations aimed at elucidating the differences in immune status between patients with SCLC and those with non-SCLC. In the present study, PBL were analyzed for T-cell surface markers, the ability to produce IL-2 and MAF, and the proliferative response to PHA and RIL-2 in order to compare the immune statuses of SCLC and non-SCLC patients.

MATERIALS AND METHODS

Subjects. The study group consisted of 18 patients with SCLC (aged 38-76 years; average age, 64.5 years; 14 males and 4 females; 1 with

stage II, 10 with stage III, and 7 with stage IV) and 15 patients with non-SCLC (5 with squamous cell carcinoma, 7 with adenocarcinoma, and 3 with large cell carcinoma; aged 38-87 years; average age, 61.8 years; 11 males and 4 females; 2 with stage I, 7 with stage III, and 6 with stage IV) (Table 1). The diagnosis was confirmed by cytological or histological examinations. The clinical staging for each lung cancer patient was based on the criteria of the International Union against Cancer-tumor, node, metastasis classification (6). None of the lung cancer patients had been given any specific medication, which might give rise to variable alteration of the hosts' immune response for a minimum of 3 months prior to entrance in the study. Two healthy volunteers and 13 patients with benign disease (aged 44-80 years; average age, 62.7 years; 11 males, 4 females) were also included in the present study as a NC control. The patients with benign disease consisted of 5 patients with cardiac disease, 3 with hypertension, 2 with Parkinson's disease, 1 with chronic obstructive lung disease, 1 with pulmonary alveolar proteinosis, and 1 with gastric ulcer.

Mice. Male BALB/c mice were purchased from Charles River Japan, Inc., Kanagawa, Japan. They were given food and water *ad libitum* and used for the experiment at an age of 7-10 weeks.

Media and Reagents. The culture medium used for incubation of PBL was RPMI 1640 medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 10% heat-inactivated human AB serum, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (Gibco Laboratories, Grand Island, NY), 2 mM L-glutamine (Flow Laboratories, North Ryde, N.S.W., Australia), 5×10^{-5} M 2-mercaptoethanol (Wako Pure Chemical Industries, Ltd., Tokyo, Japan), penicillin, 100 units/ml (Meiji Seika Co., Ltd., Tokyo, Japan) and streptomycin, 100 μ g/ml (Meiji Seika). This medium was designated PBLCM. For cultures of murine PEC, RPMI 1640 medium containing 10% heat-inactivated fetal calf serum, penicillin, and streptomycin was used and designated PCCM. The TCM consisted of RPMI 1640 medium with penicillin, streptomycin, L-glutamine, and 5% heat-inactivated fetal calf serum. PHA and TGC were from Gibco Laboratories, Detroit, MI. Ficoll-Paque was from Pharmacia Chemicals AB, Upsala, Sweden. Concanavalin A was from Sigma Chemical Co., St. Louis, MO.

Cell Lines. Friend leukemia virus-induced FBL-3 leukemic cells syngeneic to C57BL/6 mice were passed in TCM and used as target cells for cytolytic assays. A murine IL-2-dependent cytolytic T-cell line, CT6 (7), was grown in TCM containing 15% of the supernatant fluid from rat spleen cells stimulated with concanavalin A (8). The CT6 line did not possess detectable cytolytic function at the time of the present study.

Preparation of PBL. Peripheral venous blood was drawn into a heparinized syringe. Fifteen milliliters of blood were diluted 1:2 with normal saline, layered on Ficoll-Paque, and centrifuged at $400 \times g$ for 30 min at room temperature. Cells from the interface were washed 3 times with Hanks' balanced salt solution and resuspended in PBLCM at a concentration of 1×10^6 cells/ml. Cell viability was more than 95% as determined by trypan blue exclusion.

Determination of T-cell Surface Markers. Monoclonal antibodies directed against the cell surface antigens of human T-cells (OKT3), helper/inducer T-cells (OKT4), and cytotoxic suppressor T-cells (OKT8) were obtained from Ortho Diagnostic Systems, Raritan, NJ. Enumeration of peripheral blood T-cells and T-cell subsets was performed as described in (9). In brief, 100 μ l of whole peripheral blood were incubated with 10 μ l of fluorescein-conjugated monoclonal antibody at 4°C for 30 min followed by treatment with 1 ml of lysing reagent (Ortho Diagnostic) at room temperature for 5-10 min to lyse the erythrocytes, and washed 3 times with cation-free phosphate buffered saline. The antibody-coated cells were analyzed on a prototype

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³ The abbreviations used are: SCLC, small cell lung cancer; PBL, peripheral blood lymphocytes; IL-2, interleukin 2; RIL-2, recombinant interleukin 2; MAF, macrophage activating factor; NC, non-cancer; PBLCM, peripheral blood lymphocyte culture medium; PEC, peritoneal exudate cells; PCCM, peritoneal cell culture medium; TCM, tumor culture medium; PHA, phytohemagglutinin; TGC, thioglycollate; SI, stimulation index; PHA-PBL-SN, supernatants from phytohemagglutinin-stimulated peripheral blood lymphocytes; dThd, thymidine; APC, adherent peritoneal cells; TGC-APC, thioglycollate-elicited adherent peritoneal cells; 4/8 ratio, OKT4+/OKT8+ T-cell ratio.

Table 1 *Subjects in the present study*

Group	No. of cases	Cell type and no. of subjects	Age range (yr)	Sex and no. of subjects	Clinical stage and no. of subjects
SCLC	18		38–76 64.5 ± 2.3 ^a	Male, 14 Female, 4	II, 1; III, 10; IV, 7
non-SCLC	15	Squamous cell carcinoma, 5; adenocarcinoma, 7; large cell carcinoma, 3	38–87 61.8 ± 3.1	Male, 11 Female, 4	I, 2; III, 7; IV, 6
NC control	15	2 healthy volunteers, 5 with cardiac disease, 2 with Parkinson's disease, 3 with hypertension, 1 with chronic obstructive lung disease, 1 with pulmonary alveolar proteinosis, and 1 with gastric ulcer	44–80 62.7 ± 3.1	Male, 11 Female, 4	

^a Mean ± SE.

Spectrum III automated flow cytometer (Ortho Diagnostic). Lymphocytes were distinguished from monocytes and neutrophils on the basis of their 90° *versus* low forward angle light scatter. An electronic 2-dimensional analysis window was then set on the lymphocyte cluster, and its fluorescence determined as a 256-channel histogram in linear fluorescence units. An average of 2000–6000 lymphocytes/sample were analyzed. The positive cells were calculated as the percentage of PBL.

Blastogenic Response of PBL to PHA. Two hundred μl of PBL suspension (2 × 10⁵ PBL) were dispensed into each well of a flat-bottomed microtiter plate (Corning No. 25860; Corning Glass Works, Corning, NY). PHA, 0.2 μg in 10 μl PBLCM, was then added to triplicate cultures. Control cultures received supplemented PBLCM instead of PHA. After 68 h culture in 5% CO₂-95% humidified air at 37°C, 0.5 μCi [³H]dThd (25.1 μCi/mmol; Radiochemical Centre, Amersham, United Kingdom) was added to each well. After a 4-h pulse with the radiolabel, the cultures were harvested on glass fiber filters with an automatic Mash II cell harvester by using a copious amount of distilled water. The filters were air dried, and their radioactivities were counted in a liquid scintillation counter. The results were expressed as Δdpm [the arithmetic mean dpm of triplicate experimental cultures (stimulated cultures) minus the mean dpm of control cultures], and as the SI (ratio of the mean dpm of experimental cultures to the mean dpm of control cultures). In the following experiments, all incubations were performed in 5% CO₂-95% humidified air at 37°C.

Lymphokine Production by PBL. Mixtures were prepared containing 1 × 10⁶ PBL/ml in PBLCM and 1 μg PHA. One-milliliter aliquots of this mixture were incubated in the wells of Corning 25820 tissue culture plates (Corning Glass Works). PHA-PBL-SN from the cultures were collected after 48 h and stored at -20°C.

IL-2 Assay. PHA-PBL-SN were assayed for IL-2 activity essentially as described previously (8). In brief, 1 × 10⁴ IL-2-dependent CT6 cells/microwell were incubated for 44 h in the presence of serially diluted PHA-PBL-SN followed by a 4-h pulse with 0.5 μCi [³H]dThd. Cells were harvested on glass fiber filters and the [³H]dThd uptake was determined. The reciprocal of the supernatant dilution which resulted in 50% maximum [³H]dThd incorporation was defined to be the IL-2 titer expressed in units per milliliter.

Proliferative Response of PBL to Human RIL-2. Lyophilized human RIL-2, TGP-3, was generously donated by Takeda Pharmaceutical Co., Ltd., Osaka, Japan, and dissolved in 4 ml of PBLCM (10⁵ units/ml). One hundred thousand PBL/microwell were incubated for 68 h in the presence of serially diluted human RIL-2 in a flat-bottomed microtiter plate, followed by a 4-h pulse with 0.5 μCi [³H]dThd. Cells were harvested on glass fiber filters, and the [³H]dThd uptake was determined.

Preparation of Adherent Peritoneal Cell Monolayers. Murine APC monolayers were prepared as described previously (10). In brief, PEC from C3H/HeN mice given injections i.p. of 1 ml of 10% TGC solution 4 days earlier were suspended in PCCM at a concentration of 1.5 × 10⁶ cells/ml. Two hundred-μl aliquots of PEC suspension were added to round-bottomed microtiter plates (Corning No. 25850; Corning Glass Works) and incubated for 2 h. The cultures were washed 3 times with Hanks' balanced salt solution to remove nonadherent cells. The resulting adherent cell monolayers were designated TGC-APC monolayers and used as effector cells for cytolytic assays. Cytological exam-

inations revealed that more than 90% of the TGC-APC were mononuclear phagocytes (10).

MAF Assay. MAF activity was measured by the method described in detail previously (10). In brief, TGC-APC were incubated in 50% PHA-PBL-SN for 6 h. After discarding the supernatants, 3 × 10³ ¹²⁵I-labeled FBL-3 leukemic cells were added, followed by incubation for an additional 40 h in 200 μl of PCCM. One hundred μl of each supernatant were then harvested to count the radioactivity. The percentage of specific ¹²⁵I release was calculated as

$$\frac{(e - s)}{(t - s)} \times 100$$

where *e* represents the radioactivity in the supernatant from the culture containing both effector and target cells, *s* is the radioactivity in the supernatant from the culture containing only target cells, and *t* is one-half of the radioactivity of the total target cells added to each well. The spontaneous ¹²⁵I release was less than 15%. The MAF activity was expressed by the magnitude of the lytic activity of TGC-APC.

Statistical Evaluation. Statistical significance was determined by the Student's *t* test.

RESULTS

Analysis of T-cell Surface Markers of PBL from Lung Cancer Patients. At the beginning of the present study, PBL from lung cancer patients and from concurrent controls were analyzed for T-cell surface markers. The percentages of both OKT3+ and OKT4+ T-lymphocytes were a little lower in the non-SCLC group than in both the NC control group and the SCLC group. On the other hand, the percentage of OKT8+ T-cells was slightly higher in the non-SCLC group than in the other two groups (Fig. 1). Accordingly, the ratio of helper to suppressor T-cells (4/8 ratio) was a little lower in the non-SCLC group, but not in the SCLC group, compared with the NC control group (Fig. 2); however, there were no statistically significant differences in OKT3, OKT4, and OKT8 expression and 4/8 ratios between SCLC, non-SCLC, and control donors.

Proliferative Response of PBL from Lung Cancer Patients to PHA. To examine the mitogen-induced proliferative response, PBL from lung cancer patients and from concurrent controls were tested for the proliferative response to PHA. The mean Δdpm (Fig. 3A) as well as the mean SI (Fig. 3B) of the SCLC group were significantly lower than those of the NC control group. The mean SI was also significantly lower in the SCLC group than in the non-SCLC group.

IL-2 Activity in PHA-PBL-SN from Lung Cancer Patients. Although there were no quantitative changes in the T-cell subsets or the 4/8 ratio, the blastogenic response of PBL to PHA was markedly impaired in the SCLC group. Since mitogen-induced proliferation depends on production of a growth factor (11), we next determined the IL-2 activity in PHA-PBL-

IMMUNE STATUS OF SMALL CELL LUNG CANCER PATIENTS

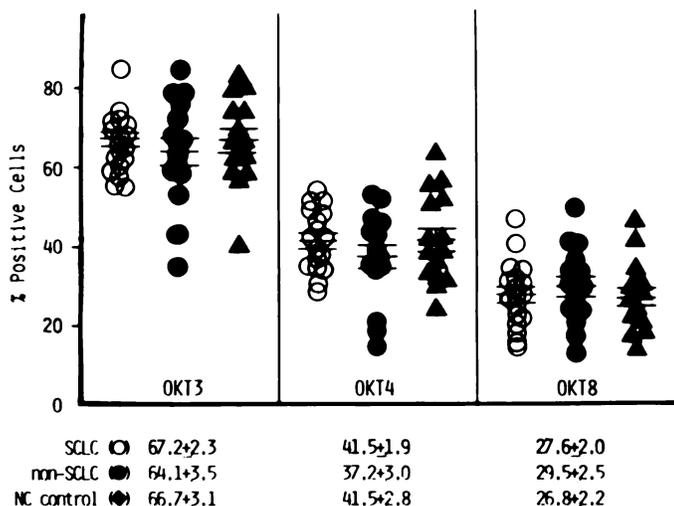


Fig. 1. Percentage of T-cell subsets in PBL from lung cancer patients. Bars, SE.

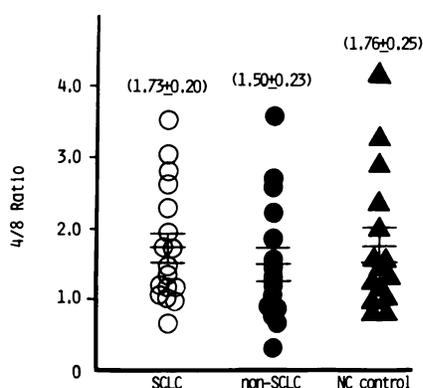


Fig. 2. The 4/8 ratios of PBL from lung cancer patients. Bars, SE.

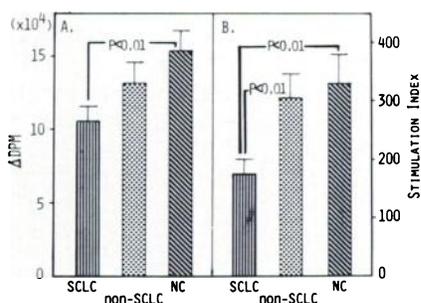


Fig. 3. Proliferative response of PBL from lung cancer patients to PHA. Two hundred thousand PBL/microwell were incubated with 0.1% of PHA for 68 h followed by a 4-h pulse with 0.1 μCi of [³H]dThd. The radioactivity incorporated into the PBL was counted. Results are expressed in Δdpm (A) and stimulation index (B). Bars, SE.

SN from lung cancer patients and from concurrent controls in order to examine the ability of PBL to produce IL-2 (Fig. 4). The IL-2 activity of the SCLC group was detectable but was significantly lower than that of the NC control group. Although not significant, the IL-2 activity was markedly lower in the SCLC group than in the non-SCLC group.

Proliferative Response of PBL from Lung Cancer Patients to Human IL-2. In addition to the inability to produce IL-2, a poor response of PBL to IL-2 was also surmised to be one of the major causes of the reduced proliferative response to PHA in the SCLC group; therefore, a further experiment was performed to compare the proliferative responses of PBL to human RIL-2 in the 3 subject groups (Fig. 5). Regardless of the

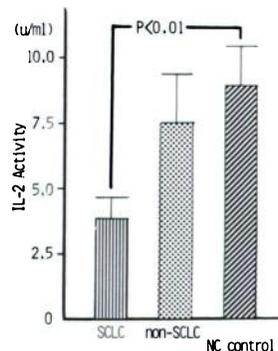


Fig. 4. IL-2 activity in the supernatants of PBL from lung cancer patients. Ten thousand CT6 cells/microwell were incubated with serially diluted PHA-PBL-SN for 44 h followed by a 4-h pulse with 0.5 μCi of [³H]dThd. The radioactivity incorporated into the cells was counted. The reciprocal of the supernatant dilution which resulted in 50% maximum [³H]dThd incorporation was defined to be the IL-2 titer expressed in units per ml. Bars, SE.

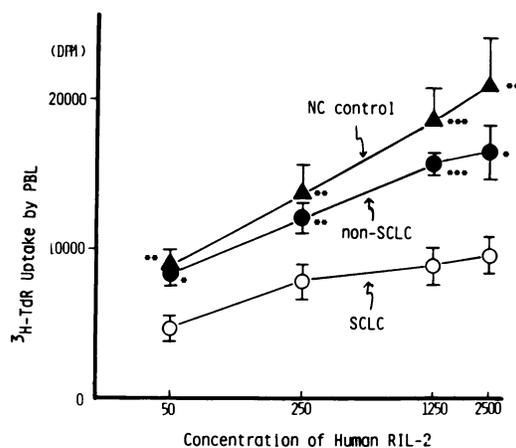


Fig. 5. Proliferative response of PBL from lung cancer patients to human RIL-2. One hundred thousand PBL/microwell were incubated in serially diluted human RIL-2 for 68 h followed by a 4-h pulse with 0.5 μCi [³H]dThd (TdR). The radioactivity incorporated in the PBL was counted. *, versus SCLC, *P* < 0.02; **, versus SCLC, *P* < 0.05; ***, versus SCLC, *P* < 0.01. Bars, SE.

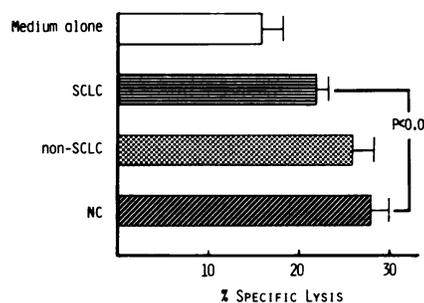


Fig. 6. MAF activity in the supernatants from PBL of lung cancer patients. TGC-APC from C3H/HeN mice were incubated with 50% PHA-PBL-SN for 6 h and tested for cytolytic activity against leukemic cells at an effector:target cell ratio of 100:1. Bars, SE.

concentration of RIL-2 added, PBL from the SCLC group demonstrated a significantly lower response to RIL-2 compared with PBL not only from the NC control group but also from the non-SCLC group.

MAF Activity in PHA-PBL-SN from Lung Cancer Patients. To determine the ability of PBL from lung cancer patients to produce another lymphokine, the MAF activity in PHA-PBL-SN was measured. As shown in Fig. 6, PHA-PBL-SN from the SCLC patients induced a significantly lower cytolytic activity in TGC-APC than did PHA-PBL-SN from the NC control group; that is, the MAF activity in PHA-PBL-SN was significantly lower in the SCLC group than in the NC control group.

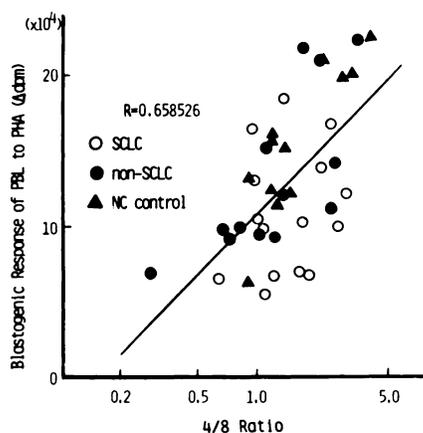


Fig. 7. Correlation between the 4/8 ratio and the blastogenic response to PHA.

DISCUSSION

Extensive studies have shown that patients with lung cancer display immune deficiency characterized by low counts of T-lymphocytes in the PBL (12), impaired reactions of delayed cutaneous hypersensitivity (13), depressed reactivities of lymphocytes to lectins (14), impaired activities of natural killer cells (15) and of antibody-dependent cell-mediated cytotoxicity (16), and altered monocyte-macrophage functions (17); however, there have been a few investigations which focused on the immune status of SCLC patients. Nielsen *et al.* (18) showed that the defect in the chemotactic activity of blood monocytes was most severe in patients with SCLC than with non-SCLC, and Shanker *et al.* (19) showed that the proportion of PBL capable of forming rosettes with sheep erythrocytes was significantly lower in cases with small cell anaplastic lung cancer than in cases with squamous cell carcinoma of the lung. Another investigation demonstrated that the chemotactic activity of blood monocytes in patients with SCLC is normalized during chemotherapy (20).

The present experiment was undertaken to help clarify the differences in immune status between patients with SCLC and those with non-SCLC. It was in the non-SCLC group rather than in the SCLC group that the percentages of both OKT3+ and OKT4+ T-lymphocytes were slightly decreased, with a slight increase in the percentage of OKT8+ T-cells and a slight decrease in the 4/8 ratio (Fig. 2), which is partially responsible for maintaining immune homeostasis. In contrast, the SCLC patients showed a significantly lower proliferative response of PBL to PHA (Fig. 3). The proliferative response to a mitogen involves a cascade of events, including production of growth factors and response to them by mature T-cells (11). IL-2 is a critical growth factor in this response. To attain optimal mitogenic responses, IL-2 must be produced and then bind to a specific receptor on the surface of responding lymphocytes; thus, poor mitogenic responses may reflect defective IL-2 production and/or altered expression of IL-2 receptors in response to stimulation. To explore the possibility that the poor proliferative response of PBL from SCLC patients to PHA could reflect poor IL-2 production or IL-2 receptor expression, PBL were analyzed for the ability to produce IL-2 and for the proliferative response to human RIL-2 as one parameter reflecting the status of IL-2 receptor expression on T-cells. The ability of PBL to produce IL-2 was markedly impaired in the SCLC patients (Fig. 4), and PBL from the SCLC patients showed a significantly lower proliferative response to human RIL-2 compared with PBL from the other 2 groups (Fig. 5). The poor

proliferative response of PBL to IL-2 may be at least partly due to reduced IL-2 receptor expression on T-cells; furthermore, the MAF activity in PHA-PBL-SN from the SCLC patients was also lower than that of the NC control group (Fig. 6). These results suggest that immunoregulatory T-lymphocytes were affected qualitatively rather than quantitatively in patients with SCLC.

The comparative analysis of immune status between the SCLC and the non-SCLC patients with stage III brought about results similar to the above (data not shown); however, no significant difference was shown between the lung cancer patients with stage III and those with stage IV (data not shown).

The present study also suggests that the balance of immunoregulatory helper and suppressor T-cells (4/8 ratio) may not reflect host immune competence. Fig. 7 shows the correlation between the 4/8 ratio and the PHA response of PBL from all of the subjects examined in the present study. As shown in Fig. 7, there is a significant correlation between them, indicating that analysis of the surface markers of the T-cells in PBL might be nonetheless a potent tool for analyzing host immune competence.

The present study has shown that host immune competence is profoundly affected in SCLC patients; therefore, rectifying the depressed immune status should be of assistance in improving the prognosis of SCLC patients. This idea is supported by the findings from a randomized controlled study that *Nocardia rubra* cell wall skeleton, which has been demonstrated to be a potent immunostimulator in numerous investigations (10, 21–24), prolongs the survival time of SCLC patients (25). The treatment of cancer patients owes its success partially to precise analysis of the immune status of the patients and on the mechanisms whereby immunodeficiency is generated, and to improving the immune response against the cancer.

The possible mechanism(s) by which immune competence is deprived in SCLC patients remains to be clarified. We are currently investigating the underlying mechanism(s).

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