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Characterization and Purification of an Immunosuppressive Factor Produced by a Small Cell Lung Cancer Cell Line

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The present study was undertaken to determine whether small cell lung cancer (SCLC) cell lines produce immunosuppressive factors and, if they do, to characterize the factors. The supernatants of SCLC cell lines, H69 and N857, inhibited not only the blastogenic response of human peripheral blood lymphocytes (PBL) to phytohemagglutinin or concanavalin A, but also the cytotoxic activity of lymphokine-activated killer cells. Neither was inhibited by supernatants from non-SCLC cell lines PC9, QG56, and A549. The immunosuppressive activity of H69 supernatant was stable upon heating to 56°C for 60 min, but labile when heated to 70°C for 10 min. The activity was abolished after dialysis at pH 2.0 or pH 11.0, but not at pH 4.5 or pH 9.0. Digestion with trypsin or proteinase eliminated the immunosuppressive activity, whereas treatment with neuraminidase, mixed glycosidase, DNase or RNase had no effect, suggesting that the immunosuppressive activity in H69 supernatant is due to a protein factor. This H69-derived immunosuppressive factor was isolated by ion exchange chromatography using a gradient of 0.04 to 0.08 *M* NaCl solution. Gel filtration and sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed the factor to have molecular weights of 98 kD and 102 kD, respectively. These results suggest that SCLC cells produce a potent immunosuppressive factor which may account for the immune deficiency in SCLC patients.

Key words: Small cell lung cancer — Immunosuppressive factor — Lymphocyte blastogenesis

Evidence has mounted in recent years that neoplastic disease is often associated with a general impairment of immune function.[1-3] High levels of serum immunoregulatory factors which nonspecifically inhibit *in vitro* and *in vivo* lymphocyte function[4-8] are thought to account for the depressed immune status in anergic cancer patients.[4,9] Furthermore, the presence of soluble immunosuppressive factors in the cell-free ascites of patients with peritoneal metastatic disease and in ascites fluids from murine tumor models has been well documented.[10-13] It has been postulated that tumor cells may locally produce or induce the formation of factors capable of inhibiting lymphocyte function, thus effecting local immunosuppression.[14-18] In the previous paper,[19] we demonstrated that small cell lung cancer (SCLC)[4] patients have a marked suppression in immunoregulatory T-lymphocyte functions, including a low proliferative response of peripheral blood lymphocytes (PBL) to phytohemagglutinin (PHA) or human recombinant interleukin 2 (rIL-2), and impaired IL-2 and macrophage-activating factor productions. Based on these findings, we postulated that SCLC cells might produce an immunosuppressive factor(s). The purpose of this investigation was to determine whether SCLC cell lines do produce immunosuppressive factors and, if they do, to analyze the characteristics of the factors.

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[4] The abbreviations used are: SCLC, small cell lung cancer; LAK, lymphokine-activated killer; PBL, peripheral blood lymphocytes.

MATERIALS AND METHODS

Media and reagents The culture medium used for maintainance of cell lines was RPMI 1640 medium (Nissui Pharmaceutical Co., Ltd., Tokyo) supplemented with the following: 10% heat-inactivated fetal calf serum; 2 m*M* L-glutamine (Flow Laboratories, North Ryde, N.S.W.); 100 units/ml penicillin (Meiji Seika Co., Ltd., Osaka); and 100 μg/ml streptomycin (Meiji Seika). This medium was designated TCM. Serum-free HITES medium constituted with RPMI 1640 medium, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 m*M* L-glutamine, 10 n*M* hydrocor tisone (Sigma Chemical Co., St. Louis, MO), 5 μg/ml insulin (Shimizu Pharmaceutical Co., Osaka), 10 μg/ml transferrin (Sigma Chemical), 10 n*M* 17β-estradiol (Sigma Chemical) and 30 n*M* sodium selenite (Shigma Chemical) was used in preparing cell culture supernatants. RPMI 1640 medium supplemented with 2% heat-inactivated human AB serum, penicillin, streptomycin and L-glutamine (designated PBLCM) was used for blastogenesis assays and lymphokine-activated killer

(LAK) cell generation. PHA was purchased from Gibco Laboratories, Detroit, MI. Ficoll-Paque was obtained from Pharmacia Chemicals AB, Uppsala. Concanavalin A (Con A), RNase, DNase, trypsin, soybean trypsin inhibitor, proteinase (Type I, crude, from bovine pancreas; specific activity, 8–10 units per mg solid), and neuraminidase were from Sigma Chemical. Mixed glycosidase was from Seikagaku Kogyo, Tokyo.

Human rIL-2 Lyophilized human rIL-2, TGP-3 (3.6×10^4 Takeda units/mg and 1.2×10^4 units/mg based on Biological Response Modifier Program reference reagent human IL-2), was generously donated by Takeda Pharmaceutical Co., Ltd., Osaka, and dissolved in PBLCM.

Cell lines Established SCLC cell lines H69 and N857 were kindly supplied by Dr. Yukio Shimosato, Pathology Division, National Cancer Center Research Institute, Tokyo. Lung adenocarcinoma cell line A549 and lung squamous cell carcinoma cell line QG56 were purchased from American Type Culture Collection, Rockville, MD. These cell lines, another lung adenocarcinoma cell line (PC9), and B cell lymphoma cell line (Daudi), were passaged twice a week in TCM.

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

Culture supernatants of cell lines Five hundred thousand H69, N857, A549, PC9 or QG56 cells were suspended in 1 ml of HITES medium. Ten milliliters of the cell suspension was incubated in a tissue culture flask (Corning No. 25100; Corning Glass Works, Corning, NY) for five days at 37°C in a 5% CO_2-95% humidified atmosphere. The supernatants were collected by centrifugation at 400*g* for 10 min, and dialyzed overnight against phosphate-buffered saline (PBS). The dialyzed supernatants were filtered through a 0.4 μm membrane filter (Sartorius GmbH, Göttingen) before use. In the following experiments, cell cultures were incubated in a 5% CO_2-95% humidified atmosphere at 37°C unless otherwise stated.

Blastogenesis assay Culture supernatants and fractions purified from the supernatants were tested for their ability to inhibit proliferation of human PBL in response to PHA and Con A as described elsewhere.[19] In brief, 200 μl of PBL suspension (2×10^5 PBL) was dispensed into each well of 96-well flat-bottomed microtiter plates (Corning No. 25860; Corning Glass Works). PHA (0.2 μg/ml in 10 μl PBLCM) or Con A (2.5 μg/ml in 10 μl PBLCM) and culture supernatants or fractionated test samples (12.5 or 25% of original concentration) were added to triplicate cultures. Control cultures received supplemented PBLCM instead of the supernatant or fractionated test sample. After a 68-h culture period, 0.5 μCi of [^{3}H]thymidine (25.1 μCi/mmol; Radiochemical Centre, Amersham) was added to each well. After a 4-h pulse with the radiolabel, cultures were harvested on glass fiber filters, and the radioactivity was determined using a liquid scintillation counter. The specific suppressive activity of supernatants of fractionated samples was defined in arbitary units as the reciprocal of the concentration of the test sample required to obtain 50% inhibition of blastogenesis.

LAK generation PBL suspension (2 ml, 2×10^6 cells) was dispensed into each well of 24-well tissue culture plates (Costar, Cambridge, MA), and incubated in the presence of 5 Takeda units/ml of human rIL-2 and 25% culture supernatant for four days. The cells were washed with PBLCM three times and assayed for cytotoxic activity against Daudi cells.

Cytotoxicity assay LAK cells were tested for cytotoxicity as described elsewhere.[20] Briefly, Daudi cells were labeled with 100 μCi of $Na_2{}^{51}CrO_4$ for 60 min. The cells were then washed three times and resuspended in PBLCM. A suspension of ^{51}Cr-labeled target cells (100 μl, 5,000 cells) was placed in each well of 96-well round-bottomed microtiter plates (Costar). LAK cells in 100 μl of PBLCM were added at various E:T ratios. Each microtiter plate was centrifuged and incubated for 4 h, and 100 μl of supernatant was counted for radioactivity. The percentage of specific cytolysis was calculated using the following formula:

$$\%\ \text{specific cytolysis} = [(e-s)/(t-s)]\times100,$$

where e represents the radioactivity in the supernatant from cultures containing both effector and target cells, s is the radioactivity in the supernatant from cultures containing only target cells, and t is one-half of the radioactivity of the total target cells added to each well. The spontaneous ^{51}Cr release was less than 15%.

Heat inactivation An aliquot of H69 supernatant was heated to 56°C in a water bath for 60 min and another aliquot was heated to 75°C for 10 min.

Effect of acid and base on immunosuppressive activity in H69 supernatant To determine the sensitivity of the immunosuppressive activity to acid or base, H69 supernatant was dialyzed for 24 h against acetate buffer (0.3 *M*, pH 2.0 or pH 4.5) or glycine buffer (0.3 *M*, pH 9.0 or pH 11.0). Dialysis was conducted in a 500-fold excess of dialysate for 24 h at 4°C. All samples were then dialyzed in a 1000-fold excess of PBS with two buffer changes per period.

Chemical treatments The susceptibility of the immunosuppressive activity to trypsin digestion was tested by the methods described elsewhere.[21] An aliquot of H69

supernatant was incubated with 100 μg of trypsin for 3 h at 37°C, then 200 μg of soybean trypsin inhibitor was added. As a control, 200 μg of soybean trypsin inhibitor was incubated with 100 μg of trypsin for 2 h at 22°C, before incubating for an additional 3 h at 37°C with the sample.

To test proteinase sensitivity, an aliquot of H69 supernatant was incubated with 20 μg/ml proteinase for 3 h. The reaction was stopped by adding 200 μl/ml of bovine serum albumin.

The supernatant was also subjected to RNase (100 μg/ml; 30 min), DNase (100 μg/ml; 30 min), mixed glycosidase (40 μg/ml; 24 h), or neuraminidase (0.01 μg/ml; 24 h) treatment at 37°C.

All samples were sterilized by filtration through a membrane filter before testing for immunosuppressive activity.

Ion exchange chromatography The crude culture supernatant (500 ml) was concentrated 20-fold by ultrafiltration through a PM-10 Diaflo (Amicon Co., Lexington, MA). The concentrated supernatant was applied to a DEAE-Sephacel column (0.9×10 cm; Pharmacia) equilibrated in 0.01 *M* Tris-HCl buffer, pH 7.6. The column was developed with a linear salt gradient (0–0.3 *M*) at a flow rate of 10 ml/h, and fractions of 3 ml were collected. Each fraction was dialyzed against RPMI 1640 medium, and 200 μl aliquots were assayed for their ability to inhibit PBL blastogenesis.

Molecular sieve chromatography The pooled fraction with the highest immunosuppressive activity from the ion-exchange step (fractions 7 to 9) was concentrated 4-fold by ultrafiltration through a PM-10 Diaflo apparatus. The Sephacryl S-300 column (1.6×98 cm; Pharmacia) was calibrated with carbonic anhydrase (Sigma), bovine serum albumin (Sigma), and aldolase (Pharmacia). The concentrated sample was applied to the column by the method previously described.[22] Buffer flow rates were maintained at 15.0 ml/h, and 2.5 ml samples were collected throughout the fractionation process. Each fraction was dialyzed against RPMI 1640 medium and assayed for immunosuppressive activity.

Electrophoresis on SDS-polyacrylamide gels A modification of the procedure of Laemmli[23] was used. Sodium dodecyl sulfate was used as a dissociating agent and 2-mercaptoethanol as a reducing agent. Gel filtration fractions with the highest immunosuppressive activity were concentrated 60-fold and combined with an equal volume of Laemmli's buffer. Samples were run on a 7.5% acrylamide resolving gel in a Tris-glycine-SDS buffer. The gels were then fixed in 50% methanol, washed with water and 10% glutaraldehyde, and stained with a silver staining kit (Wako Pure Chemical Industries, Ltd., Osaka).

Suppressive factor elution from SDS-polyacrylamide gels The gel was transferred to a glass plate, kept cold, and cut with a scalpel into 2-mm slices. Each slice was crushed by passing it through a 19-gauge needle, and incubated for 24 h at room temparature in a small volume of PBLCM. Eluates were dialyzed for 36 h against a 1,000-fold excess of RPMI 1640 medium and tested for suppressive activity.

Protein determination The protein content of culture supernatants or fractionated samples was determined by the Coomassie blue technique (Bio-Rad Laboratories, Richmond, CA).

RESULTS

Effect of supernatants on blastogenesis The effect of the supernatants from various lung cancer cell lines on PHA- or Con A-induced PBL blastogenesis was determined (Table I). Both the proliferation of PBL induced by PHA and that induced by Con A were markedly inhibited by supernatants of SCLC cell lines H69 and N857. In particular, H69 supernatant showed a potent inhibitory activity. On the other hand, no suppressive activity was observed in the supernatants of non-SCLC cell lines PC9, A549, and QG56. Furthermore, none of the supernatants from 10 human or murine non-SCLC

Table I. Effect of SCLC Supernatants on T-Cell Proliferation[a]

Supernatant	Mitogen	[^{3}H]thymidine uptake (dpm)	
		Concentration of supernatant	
		25%	12.5%
PHA response			
H69	+	1,962±176[b]	9,763±515
N857	+	48,264±7,434	222,929±6,923
PC9	+	276,167±7,167	231,982±19,084
QG56	+	249,607±5,318	195,813±2,427
A549	+	204,453±9,729	175,340±2,963
—	+	233,481±15,367	
—	−	610±91	
Con A response			
H69	+	1,016±141	4,822±377
N857	+	30,414±956	127,257±2,564
PC9	+	186,469±2,721	144,582±6,192
QG56	+	146,268±3,974	124,255±2,215
A549	+	117,631±4,871	114,009±1,232
—	+	110,788±1,750	
—	−	610±91	

a) Human PBL were incubated with 0.2 μg/ml PHA or 2.5 μg/ml Con A in the presence of the supernatants from various lung cancer cell lines for 68 h, followed by a pulse with 0.5 μCi of [^{3}H]thymidine for 4 h.
b) Mean±SE of the triplicate determinations.

tumor cell lines showed as potent an immunosuppressive effect as H69 supernatant (data not shown).

Effect of supernatants on LAK generation LAK cells were generated in supernatant (25% of original concentration) from various lung cancer cell lines and tested for cytotoxic activity against Daudi cells (Table II). Both H69 and N857 supernatants markedly suppressed the cytotoxic activity of LAK cells, whereas LAK cells induced in the supernatants of non-SCLC cell lines maintained a potent cytotoxic activity at any E:T ratio. In the following experiment, H69-derived immunosuppressive factor, which showed the most potent immunosuppressive activity, was physicochemically characterized.

Table II. Inhibition of LAK Induction by SCLC Supernatants[a]

Supernatant	IL-2	% Specific lysis, E:T ratios 40:1	% Specific lysis, E:T ratios 20:1
—	−	1.1±0.2[b]	1.0±0.5
—	+	72.7±0.7	55.3±1.2
H69	+	15.6±0.6	10.9±1.3
N857	+	21.7±1.1	17.4±1.3
PC9	+	84.4±1.8	79.4±2.5
QG56	+	80.5±1.8	72.6±1.6
A549	+	83.7±0.9	79.2±1.9

a) Human PBL were incubated with 25% of supernatants from various lung cancer cell lines in the presence of 5 U/ml of human recombinant IL-2 for 4 days, and assayed for cytotoxic activity against Daudi cells at the E:T ratios indicated.
b) Mean±SE of the triplicate determinations.

Table III. Physicochemical Properties of the Immunosuppressive Active Factor in H69 Supernatant

Treatment	Suppressive activity[a] (U/ml)
Heating	
No treatment	6.8
56°C, for 60 min	6.0
75°C, for 10 min	2.5
Acid or alkaline treatment	
No treatment	6.8
pH 2.5	1.1
pH 4.5	7.1
pH 9.0	8.7
pH 11.0	1.0
Enzyme treatment	
No treatment	6.3
DNase (100 μg/ml)	5.3
RNase (100 μg/ml)	5.6
Mixed glycosidase (40 μg/ml)	5.4
Neuraminidase (0.01 U/ml)	5.4
Trypsin (10 μg/ml)	0.0
Proteinase (20 μg/ml)	3.6

a) The suppressive activity was defined in arbitary units as the reciprocal of the concentration of the test sample required to obtain 50% inhibition of blastogenesis.

Heat inactivation The suppressive effect of H69 supernatant was unaffected by heating to 56°C for 60 min. However, significant loss of suppressor activity occurred after incubation at 75°C for 10 min (Table III).

Sensitivity to acid or base Dialysis of H69 supernatant against acetate buffer (pH 2.5) and glycine buffer (pH 11.0) resulted in a nearly complete loss of the capacity to suppress PHA-induced proliferation of PBL (Table III). On the other hand, treatment at pH 4.5 or pH 9.0 had no effect on the immunosuppressive activity of H69 supernatant.

Enzymatic digestion Supernatants of H69 were exposed in separate experiments to DNase, RNase, mixed glycosidase, neuraminidase, trypsin and proteinase, and were then tested for their ability to inhibit PHA-induced blastogenesis of PBL (Table III). Treatment with DNase, RNase, mixed glycosidase and neuraminidase failed to suppress the activity of H69 supernatant. However, a significant loss of suppressor effect occurred after treatment with proteinase, and treatment with trypsin resulted in a complete loss of the capacity to suppress PHA-induced proliferation.

Ion exchange chromatography Crude culture supernatant of H69 was applied to an ion exchange column, and eluted with a salt gradient (Fig. 1). One peak of immunosuppressive activity was detected at a salt concentration of 0.04 to 0.08 *M* (Fractions 7 to 9). These fractions were pooled for further gel filtration chromatog-

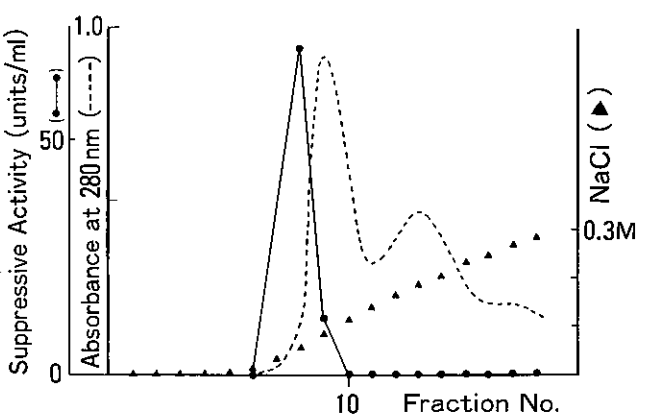

Fig. 1. Analysis of serum-free H69 supernatant by DEAE-Sephacel ion exchange chromatography. A linear gradient of 0 to 0.3 *M* NaCl at a flow rate of 10 ml/h was used. Each fraction was tested for immunosuppressive activity.

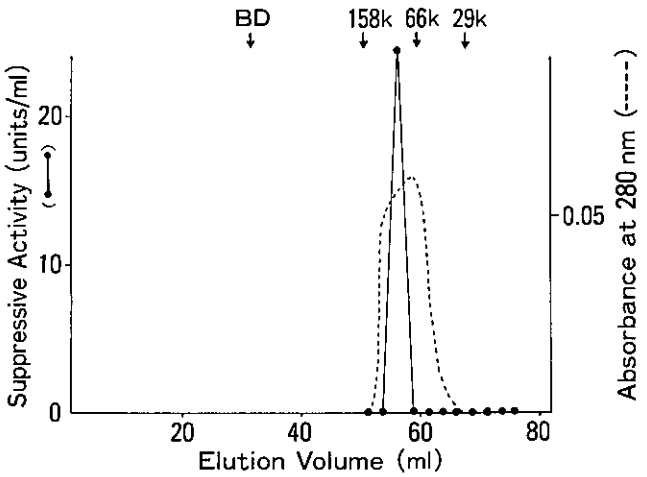

Fig. 2. Gel filtration chromatography of H69 supernatant. Fractions 7 to 9 obtained by DEAE-Sephacel ion exchange chromatography, and concentrated 4-fold with an Amicon filter, was passed through a column (1.6×98 cm) of Sephacryl-S 300 equilibrated with 0.05 *M* NaCl and 0.01 *M* phosphate buffer. Fractions of 2.5 ml were collected at 15 ml/h at 4°C, and were tested for immunosuppressive activity. The protein markers used to calibrate the column were carbonic anhydrase (29,000 daltons), bovine serum albumin (66,000 daltons), and aldolase (158,000 daltons).

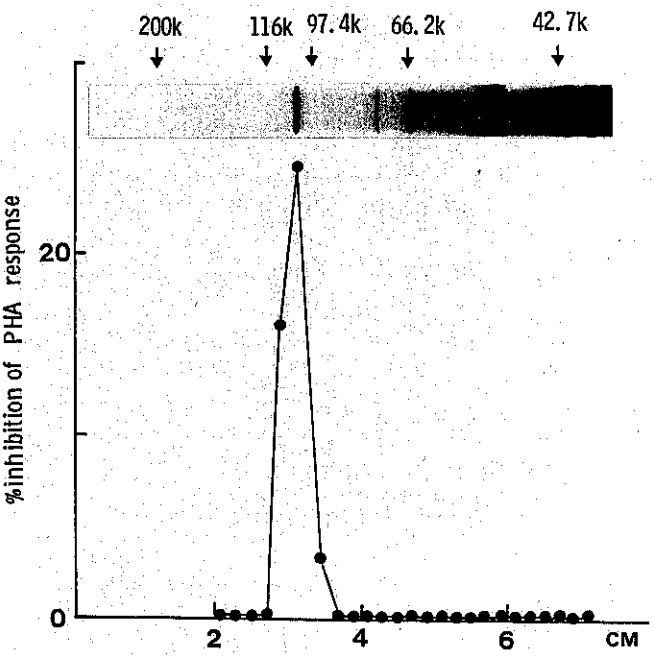

Fig. 3. Elution of H69 suppressive factor from SDS-polyacrylamide gel. To prove that the M.W. 102,000 band represents suppressive activity, the SDS-polyacrylamide gel was cut into 2-mm slices. Each slice was eluted with PBS and tested for immunosuppressive activity. A single peak of activity was associated with a M.W. 102,000 band.

raphy. A potent immunosuppressive activity was also detected in the fraction eluted by 0.04 to 0.08 *M* NaCl solution from the other SCLC cell lines, N857 and N231, but not from non-SCLC cell line, PC9 (data not shown).

Gel filtration The pooled, semipurified immunosuppressive fraction were chromatographed on Sephacryl S-300. Fig. 2 shows the elution profile. Esch fraction was assayed for immunosuppressive activity. The suppressor activity was found exclusively in fraction 23, corresponding to an approximate molecular weight of 98 kD.

Electrophoresis on SDS-polyacrylamide gel The active fraction obtained by gel filtration was concentrated 60-fold, and aliquots were run on a 12.5% SDS-polyacrylamide gel (Fig. 3). One lane was stained while the others were cut into 2-mm slices. Each slice was crushed and eluted for 24 h in a small volume of PBLCM. The eluate from each gel slice was tested for ability to inhibit PHA-induced blastogenesis. The majority of the immunosuppressive activity was associated with the 102 kD band.

DISCUSSION

It has been well documented that patients with malignant tumors have a compromised immune status.[1-3] Extensive studies have also shown that lung cancer patients display an immune deficiency.[24-29] In a previous study,[19] we demonstrated that SCLC patients have marked suppression of immunoregulatory T-lymphocyte functions. The present study was performed to characterize and semi-purify the immunosuppressive factors produced by SCLC cell lines.

Supernatants from the SCLC cell lines inhibited not only PHA- and Con A-induced proliferation of human PBL, but also the cytotoxic activity of LAK cells to a much greater degree than did those of the non-SCLC cell lines. The supernatants had been prepared in serum-free medium, suggesting that serum-derived factors are not responsible for the immunosuppressive effects of supernatants from SCLC cell lines.

Supernatants of H69 strongly inhibited the expression of Tac antigen on PBL stimulated with PHA (data not shown), indicating that SCLC supernatants suppress the immune reaction through an inhibition of IL-2 receptor expression. H69 supernatants had no effect on the proliferation or viability of PBL or IL-2-dependent cell line CT6 in the presence of human rIL-2. This confirmed that H69 supernatant had no toxic effect against PBL and CT6 cells.

Prostaglandin E_2 is known to inhibit various immune reactions.[30-32] Although prostaglandin E_2 was detected in the supernatants of the SCLC and non-SCLC cell lines, its concentration did not correlate with the inhibitory activity against PHA-induced blastogenesis of PBL. This

suggests that the immunosuppressive factor in H69 supernatant is different from prostaglandin E_2. Transforming growth factor-β (TGF-β) has also been reported to suppress immune response.[33] It is a polypeptide with a molecular weight of 25 kD and is stable to acid and base. Various tumor cells produce a substance associated with a TGF-β-like function. Wrann *et al.*[34] have shown that the T-cell suppressor factor produced by human glioblastoma cells is closely related to TGF-β. However, there has been no report demonstrating that SCLC produces TGF-β or TGF-β-related substances. Furthermore, the physicochemical properties of the H69-derived immunosuppressive factor suggest that it may be different from TGF-β. Medoff *et al.*[12] detected an immunosuppressive factor in cancerous ascites and analyzed its properties. It has a molecular weight of 50 kD. Its activity remains unaffected by treatment at pH 2.0 or at pH 10.0, or with trypsin, and remains stable upon heating to 70°C for 30 min. Werkmeister *et al.* [17] demonstrated that a human melanoma cell-derived factor inhibits the mitogenic response of lymphocytes to PHA. This factor is a glycoprotein with a molecular weight of 200 kD. The immunosuppressive factor produced by adult T-cell leukemia has been shown to have a molecular weight of 50 kD.[35] Its activity is destroyed by treatment at pH 2.3 but not at pH 9.0, or with trypsin. Colon cancer cell lines produce a soluble substance that blocks mitogen-induced T-cell proliferation and the production of IL-2.[36] The factor has an apparent molecular weight of 56 kD and an isoelectric point of 7.9. It is sensitive to endopeptidase, heating to 56°C, and exposure to pH 3.5 and pH 9.5. These immunosuppressive factors are not similar to H69-derived immunosuppressive factor in their physicochemical properties, suggesting that the H69-derived factor may be a novel immunosuppressive factor. Furthermore, potent immunosuppressive activity was detected in the fractions eluted by ion exchange chromatography from the supernatants of SCLC cell lines N231 and N857, but not from the supernatants of lung adenocarcinoma cell line PC9 (data not shown). These findings suggest that the H69 immunosuppressive factor may be specifically produced by SCLC cells. The present study is the first to demonstrate that SCLC cells produce a potent immunosuppressive factor. Additionally, study results suggest that the immunosuppression is generated in SCLC patients through the production of the immunosuppressive factor by SCLC itself.

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