A cell line (KSNY) in vitro, which produces colony-stimulating activity (CSA) for human and mouse marrow cells, has been established. A biopsy was performed on the tumor mass of a lung cancer patient who had developed extensive leucocytosis. A piece of the tumor was transplanted to a nude mouse. The secondarily transplanted mice, in turn, developed extensive leucocytosis. The mouse tumor was then removed and placed in culture bottles. To date, the KSNY cells have been maintained in vitro continuously for 15 months. By the use of a methylcellulose bone-marrow colony-formation technique, a high level of CSA in the supernatant of the tumor cell culture was recognized. Doubling time of the cell line is 46 hr. The modal chromosome number is 52, ranging from 45 to 106.

The technique of granulocytic colony formation in vitro has been widely applied to studies on granulopoiesis. It is known that colony-stimulating activity (CSA), which is necessary for clonal granulocytic differentiation, is present in various conditioned media or in tissue extracts but it is rather difficult to obtain constantly voluminous CSA with high activity. This report deals with the establishment and characteristics of a CSA-producing cell line (KSNY) from a tumor of lung cancer patient.

Materials and Methods
Establishment of the Cell Line: A 58-year-old male patient with lung cancer developed marked leucocytosis (leucocytes 78,000/mm³, mature neutrophils 90%) in May, 1977. The biopsied tumor was poorly differentiated squamous cell carcinoma histologically. A part of the tumor mass obtained was washed twice with α-minimum essential medium (α-MEM, Flow Laboratories, U.S.A.) with 20% fetal bovine serum (FBS, Flow Laboratories) and 100 μg/ml of kanamycin, minced to small pieces (approximately, 3 × 3 mm). A piece was then transplanted subcutaneously into a nude mouse. At the second month after transplantation, the tumor was resected, minced to about 3 × 3 mm, and retransplanted in three nude mice. On March 28, 1978, a tumor was removed and cultured in MA30 culture bottles (Ikemoto Co., Tokyo) containing α-MEM with 20% FBS. One-half volume of the medium was changed every week for the first month using α-MEM with 20% FBS, and twice a week from the second month on using α-MEM with 10% FBS.

Chromosomes: Chromosomes were checked by the method of Rothfel and Siminovitch, and the G-banding technique was used for identification of chromosomes.

KS NY Cell Proliferation: Concentration of 5 × 10⁴ or 10⁵ cells/ml was cultured in α-MEM containing 10% FBS and 100 μg/ml of kanamycin.
in short culture tubes. The medium was changed every 2 days. Cell number was counted at days 2, 4, and 7, using the citric acid-Crystal Violet staining.

Plating Efficiency: Four cultures of $5 \times 10^5$ cells were plated in 60-mm Falcon petri dishes (Falcon #3002, 60 × 15 mm) containing α-MEM with 10% FBS, and maintained for 7 or 14 days at 37° in a humidified atmosphere with 5% CO₂.

Conditioned Medium: Cultures of $4.8 \times 10^6$ cells of L (originating from mouse fibroblasts), KONT (another CSA-producing cell line5)), and KSNY cell lines were maintained in TD-40 culture bottles (Ikemoto Co., Tokyo) containing α-MEM with 20% FBS. The supernatant of the cultures was harvested on day 7.

Human leucocyte conditioned medium (HL-CM) for colony formation of human cells was prepared, using a modification of the method of Iscove et al.4) Several conditioned media were then centrifuged at 1,700g for 10 min, filtered through a 0.45-μm Millipore filter, and stored at −20°. Standard HL-CM and L cell-conditioned medium (L-CM) were used as controls for human and mouse cell colony formation, after being proved to have a sufficient level of CSA.

Colony Formation Procedure: In colony formation, slight modification of the method of Worton12) was used. Briefly, $5 \times 10^4$ C3H/He mouse nucleated bone marrow cells or $2 \times 10^5$ human non-adherent cells were plated in a 35-mm plastic petri dish (Lux, 35 × 10 mm) with 1 ml of the medium containing α-MEM, FBS (20%), CM (20%), and 0.88% methylcellulose (4,000 centipoises, Dow Chemical, U.S.A.). To avoid an endogenous CSA which is produced from adherent cells, non-adherent population (NA cells) were separated by the glass adherence technique described by Messner et al.6) The cultures were then incubated at 37° in a humidified atmosphere with 5% CO₂. At day 7 for mouse and at day 14 for human, colonies containing more than 20 cells were counted using an inverted microscope.

Results and Discussion

Four months after the retransplantation, the leucocyte level increased to 90,000/mm³. In order to measure the number of granulopoietic progenitor cells (CFU-C) in the retransplanted nude mouse, bone marrow colony formation was carried out. One hundred and sixty colonies per $5 \times 10^4$ bone marrow cells were observed in a plate containing our standard L-CM. This was substantially higher than the normal range of approximately 100±20 colonies.

Within 2 or 3 weeks after removal from the nude mouse, a few epithelial-like cells, together with mouse fibroblast-like cells, adhered to the bottom of the culture bottles. After the first month, the fibroblast-like cells disappeared.

Photo 1 is of a full sheet of KSNY cells at the end of the 2nd month. The cell size varied, and numerous granules were noted in the cytoplasm. Large nuclei and poly-nuclei, as well as mono- and poly-nucleoli were observed. Photo 2 shows KSNY cells at the end of 10 months. The cell size has become uniform and granules can also be detected in some of the cells.

Chromosomes were studied at the end of the 2nd and 10th month. Karyotypic analysis revealed the KSNY cell line to be a human aneuploid type. The modal chromosome number of the second sample was 52 in 200 cells examined, with a range of 45 to 106. Two common markers (mar 1 and mar 8) and several uncommon markers were presented in G-banding analysis. Mar 1 was

<table>
<thead>
<tr>
<th>CM</th>
<th>Mouse colonies/5 × 10⁴ cells (mean ± SD)</th>
<th>Human colonies/2 × 10⁵ non-ad. cells (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Case 1</td>
</tr>
<tr>
<td>None</td>
<td>0</td>
<td>5.3 ± 2.3</td>
</tr>
<tr>
<td>L-CM</td>
<td>148.0 ± 11.3</td>
<td>25.4 ± 5.6</td>
</tr>
<tr>
<td>HL-CM</td>
<td>3.4 ± 2.1</td>
<td>95.0 ± 9.5</td>
</tr>
<tr>
<td>KONT-CM</td>
<td>76.5 ± 3.5</td>
<td>168.2 ± 3.0</td>
</tr>
<tr>
<td>KSNY-CM</td>
<td>69.5 ± 6.5</td>
<td>89.5 ± 4.5</td>
</tr>
</tbody>
</table>

Table I. Comparison of CSA in Conditioned Media
larger than the No. 1 chromosome and arm ratio was 7, and mar 8 was a smaller metacentric chromosome than the G-chromosome and the arm ratio was about 1.

In an examination of KSNY cell growth during the 6th month, $5 \times 10^4$ cells had proliferated up to 8.3 times at the end of day 7. The doubling time was estimated from the growth to be approximately 46 hr. The plating efficiency of KSNY cells in the 6th month was 7%.

The results of CSA assay of KSNY-CM are shown in Table I. With the use of $2 \times 10^5$ human non-adherent cells as a target, 90 colonies were counted in Case 1 and 81 colonies in Case 2 in the presence of KSNY-CM, compared with 95 and 61, respectively, in the presence of HL-CM, and only a few colonies in the absence of CM. Therefore, the CSA level in KSNY-CM was thought to be approximately the same as that in our standard HL-CM, whereas, by the use of KONT-CM with a high level of CSA, as many as 168 and 146 colonies, respectively, were recognized. In mouse experiments, 70 colonies were observed in a plate containing KSNY-CM, while no colonies were detected in the absence of CM. In other experiments, KSNY-CM showed essentially the same results as in Table I.

Recently, several human CSA-producing cell lines in vitro have been reported. Di Persio et al. isolated two human monocyte-like cell lines from a monocyte-enriched fraction of normal blood and from a lung metastasis of fibrous histiocytoma. T3M-1 by Okabe et al. originated from a human squamous cell carcinoma of the oral cavity. In addition, KONT was isolated from the pleural effusion of a lung cancer patient (giant cell carcinoma) by Kimura et al. These findings suggest that various kinds of tumors have CSA-producing capability. It is especially interesting that KSNY-CM and standard HL-CM contain the same level of human CSA. Moreover, KSNY-CM can be obtained consistently and voluminously.

KSNY cells are therefore very useful for research on the mechanism of granulopoiesis.

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REFERENCES
EXPLANATION OF PLATE

Photo 1. Morphological features of the monolayer-cultured cells (KSNY), 2 months after isolation from the tumor of a nude mouse. The fibroblast-like cells of the nude mouse disappeared. The cell size was varied. Large and poly-nuclei, as well as mono- and poly-nucleoli are observed. Inverted microscope. ×290.

Photo 2. Inverted microscopic observation of a monolayer of KSNY cells at 10 months after isolation. The cell size has become uniform. The proliferation pattern of the cells has been recognized as the epithelial type. ×290.