Growth Characteristics and Drug Responses of a Murine Lung Carcinoma in Vitro and in Vivo

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ABSTRACT

Cells obtained from the Nettesheim lung carcinoma of DBA/2 mice, a heterogeneous population grown s.c., were cultured as monolayers. These cells were serially subcultured and cloned twice, and a clone was selected for further study. This clone produced malignant tumors at the injected site when injected s.c. into male DBA/2 or C57BL/6 × DBA/2 F, mice. Referred to as KLN205, this cell line had the highest rate of lung colony formation on i.v. injection. It was subcultured for over 15 generations, and its cytological characteristics were investigated. The s.c. and lung colony growth were examined histologically.

The effects of treatment with two antimetabolite drugs, arabinosyl-6-mercaptopurine (NSC 406021) and 6-selenoguanosine (NSC 137879) were determined in culture and in vivo. The former was relatively ineffective; the latter was very effective both in vivo and in vitro. Several drugs used clinically for the treatment of lung cancer were also tested. This established and characterized cell line is proposed as a potential model for testing other chemotherapeutic treatments.

INTRODUCTION

Primary lung carcinomas have become the most frequent cause of cancer fatalities in men and the third commonest cause in women. These fatality rates are still increasing, and such cancers have not been very responsive to presently available chemotherapeutic agents. In addition, many other neoplasms metastasize to the lungs. The selection of new drugs for lung cancer therapy might be aided by the availability of a suitable animal model or models resembling the human disease. A squamous cell carcinoma of the lung in DBA/2 mice obtained by Nettesheim and Hammons (5) appeared to have some desired characteristics. However, this tumor was composed of heterogeneous cell populations (7). It would be advantageous to have a homogenous population and 1 that could be readily tested for response to drugs in vitro and in vivo. We have established from this tumor a cloned cell line that will grow in cell culture and can be transplanted s.c. or i.v. to male DBA/2 or C57BL × DBA/2 F, (hereafter called BD2F1) mice where it has reproducible rates of growth and kills the mice. Tests with 2 antimetabolites, ara-6MP and SeGR, that are effective in some other experimental systems (1–4, 6) showed that the former was only marginally effective against the lung carcinoma at the doses used and that the latter was very effective. The results in vivo paralleled those in vitro. Several drugs in common clinical use for the treatment of lung cancers were also tested against this model system.

MATERIALS AND METHODS

The Nettesheim carcinomas were initially transplanted as approximately 1- to 2-mm blocks s.c. into male DBA/2 mice supplied by our central breeding facility from genetically controlled breeders obtained at The Jackson Laboratory, Bar Harbor, Maine. The s.c. tumors were dissected out aseptically after 2 weeks of growth and placed in sterile petri dishes with sterile Ca2+-free PBS. The tumor masses were cut into fragments with scissors and forced through a 200-mesh stainless steel screen with a pestle. The suspensions were treated twice in PBS containing 0.25% pancreatin (Grand Island Biological Co., Grand Island, N. Y.) for 15 min at room temperature. The suspensions were further treated in PBS containing 0.25% trypsin-EDTA (Grand Island Biological Co.) for 15 min at room temperature. The cells were resuspended once in trypsin-free PBS and centrifuged and washed twice by resuspension in MEM + 10% FCS (Grand Island Biological Co.). The cells were resuspended in MEM + NEAA + 10% FCS + gentamicin, 50 μg/ml (Schering Corp., Kenilworth, N. J.), + Fungizone, 2.5 μg/ml (Grand Island Biological Co.), + 10% conditioned medium. The conditioned medium was obtained from cultures of L1210 cells and filtered through millipore filters (Millipore Corp., Bedford, Mass.) of 0.22-μm pore size. The cell suspension was distributed in Conning culture flasks (10 ml) and incubated at 37° in an atmosphere of 5% CO2:95% air. The medium was changed weekly until sufficient monolayer growth was obtained. For subcultures the monolayers were rinsed once with 0.9% NaCl solution and incubated with 0.25% trypsin:EDTA at 37° under 5% CO2:95% air for 10 to 15 min. The cells were dispersed and washed once with MEM + NEAA + 10% FCS. The cells were centrifuged, the pellet was resuspended in the same medium and counted in a Coulter counter, and subcultures were inoculated with 1 to 5 × 10⁴ cells/10 ml.

Clones were obtained by seeding 400 to 800 cells in a Corning culture plate (10 ml), incubating for 2 to 4 weeks, and removing well-isolated colonies with cloning rings. Each clone was repeatedly subcultured as described earlier until consistent growth was obtained. The tumorigenesis of each clone was tested by dispersing the monolayer cells as described for subculturings and inoculating them into DBA/2 or BD2F1 mice either s.c. or i.v.

For determination of growth rate, culture flasks were...
each inoculated with $3 \times 10^6$ cells in 10 ml of MEM + NEAA + 10% FCS and incubated at 37°C in 5% CO$_2$:95% air. Flasks were taken out every day; cells were dispersed as described earlier and counted in a Coulter counter. The medium was changed every 3 days. The population-doubling time was determined from the growth curve. Male BD2F, mice were chiefly used for lung colony assay with i.v. inoculation because Nettesheim carcinoma cells tend to show better transplantability in male than in female BD2F, mice (P. Nettesheim, personal communication). The mice were sacrificed 3 weeks after transplantation and lung colonies were counted except where otherwise noted.

Determination of numbers of lung tumors was carried out as follows. Mice were anesthetized with penthrane (Abbott Laboratories, Montreal, Quebec, Canada) and sacrificed by severing the aorta and withdrawing as much blood as possible. Fixative, 1 to 1.5 ml [formaldehyde:glacial acetic acid:95% ethanol, 5:5:90 (v/v)] was injected into the trachea, and the lungs were expanded. The expanded lungs were removed and placed in a bottle containing the fixative for 24 hr. Lung specimens were cleared essentially by the technique of Yuhas (8). That is, they were washed twice with 70% ethanol; stained in 50% ethanol containing 4% Diene's stain (Grand Island Biological Co.); rinsed twice in 70% ethanol, twice in 95% ethanol, and twice in absolute ethanol; partially “cleared” overnight in oil of cedarwood (J. T. Baker Chemical Co., Phillipsburg, N. J.); and finally cleared by soaking in methyl salicylate (J. T. Baker). The lung colonies were counted under a dissecting (x10) microscope.

When microspheres (3M Co., Nuclear Products, 3M Center, St. Paul, Minn.) were used in cell suspensions for i.v. injection, they were washed once with 0.9% NaCl solution and sonically disrupted before being mixed with the cells.

Cells grown in vitro were examined after staining with Wright's stain (200 mg in 100 ml methanol; Allied Chemical Co., Morristown, N. J.), on the glass for 4 min and then diluted with 2 ml 5 mM KH$_2$PO$_4$ (pH 6.8) for 8 min and rinsed with water.

SeGR (NSC 137679) was obtained from Drug Research and Development, National Cancer Institute, Bethesda, Md. ara-6MP (NSC 406021) was obtained from Pfanstiehl Laboratories, Inc., Waukegan, Ill. The suppliers of other drugs tested were: 1-(2-chloroethyl)-3-(trans-4-methyl-cyclohexyl)-1-nitrosourea, Drug Research and Development, National Cancer Institute; Adriamycin, Adria Laboratories Inc., Wilmington, Del.; Cytoxan, Drug Research and Development, National Cancer Institute; hexamethylmelamine, Drug Research and Development, National Cancer Institute; methotrexate, Cyanamid of Canada, Ltd., Montreal, Quebec, Canada; thioguanine, Dr. G. H. Hitchings, Burrows-Welcome Co., Durham, N. C.; and 5-fluorouracil, Sigma Chemical Co., St. Louis, Mo.

RESULTS

The s.c. growths of the Nettesheim tumor were collected from 25 male DBA/2 mice, the cells were dispersed, and 25 Corning culture flasks were inoculated with $5 \times 10^5$ to $5 \times 10^6$ cells/flask, each containing 10 ml of medium. Several flasks were discarded because of contamination or because cell development was mostly fibroblastic. Cells from the latter flasks did not develop tumors when reinoculated into DBA/2 mice.

Three flasks given $5 \times 10^5$ cell inocula slowly developed monolayers of epithelial-like cells in the course of about 1 month. These cells produced tumors when inoculated into DBA/2 mice. The cultured cells have grown faster from the second generation and have been subcultured every 10 to 14 days with changes of medium made 1 to 3 times/week. The cells were subcultured 15 times, and cloning was carried out twice with 1 of the lines. The second cloning yielded 8 colonies. Each colony was subcultured and compared for tumorigenesis. The lung colony-forming ability of the cells of each clone was tested as follows: male BD2F, mice were given i.v. injections of $5 \times 10^5$ cells/mouse, and the mice were sacrificed and checked for lung nodules after 3 weeks. The numbers of mice with tumor nodules per group of 5 mice inoculated were 2 of 5, 0 of 5, 5 of 5, 3 of 5, 5 of 5, 5 of 5, 2 of 5, and 4 of 5 for the 8 clones. Thus 3 clones were indicated to be highly transplantable. A clone designated KLN205 was selected for further work because it grew relatively rapidly in vitro.

This cell line was also able to produce lung colonies in male DBA/2 mice when injected i.v. The cell line also was established as transplantable to male DBA/2 and BD2F, mice s.c.

Those mice inoculated with $2 \times 10^6$ or $5 \times 10^6$ cells s.c. or i.v. died within 6 to 8 weeks. To obtain data on lung metastases from s.c. tumors, $3 \times 10^5$ cells/mouse were transplanted s.c. to 5 DBA/2 and 5 BD2F, male mice. The mice were sacrificed after 1 month and examined for lung metastases. Two of 5 DBA/2 mice and of 5 BD2F, mice had lung tumor nodules. These were less than 1 mm in diameter. Fig. 1 shows a stained preparation of KLN205 cells grown in vitro. The cells shown were from a 3-day growth in a flask inoculated with $3 \times 10^6$ cells/10 ml. Staining was with Wright’s stain. The cells formed a pavement-like monolayer. Occasional cells showed double nuclei, and the number and size of nucleoli differed from cell to cell.

A growth curve for cell cultures is shown in Chart 1. Cells of the 16th passage in culture were subcultured at $3 \times 10^6$ cells/10 ml. Three flasks were removed each day at the

![Chart 1. Growth curve of KLN205 cells in vitro. Culture flasks were each inoculated with $3 \times 10^6$ cells in 10 ml medium. Flasks were taken out every day; cells were dispersed and counted in a Coulter counter. Points, average number of cells per flask from triplicate cultures.](image-url)
same time and trypsinized as described previously, and cells/flask were counted in a Coulter counter. Each point on the curve is the average of counts from 3 flasks. The calculated doubling time was 31 hr.

The number of single cells in a typical suspension (as contrasted to clumps) was determined. A 6-day culture of KLN205, started with a $3 \times 10^6$ cell inoculum, was dispersed as described. The dispersed cells were washed once with medium and resuspended in calcium-free PBS. When a drop was examined on a slide and 400 cells were counted under the microscope, samples from 6 flasks showed that $85.8 \pm 1.03\%$ (S.E.) were single cells.

The s.c. tumors are composed of poorly differentiated epidermoid carcinoma cells with numerous mitotic figures (Fig. 2). The tumors show evidence of keratinization, and there are frequently extensive areas of necrosis, located centrally and containing heavy polymorphonuclear infiltration. At the periphery there is a narrow discontinuous zone of lymphocytic infiltration and fibrosis.

Cell suspensions, $3 \times 10^6$ cells and $5 \times 10^5$ microspheres in 0.5 ml/mouse, were injected in the tail veins of male BD2F1 mice. The mice were sacrificed at 4 weeks, and the lungs were prepared as described earlier. Fig. 3 shows such lung preparations with dark spots representing tumor nodules in the relatively transparent lung parenchyma. The histology of such nodules was very similar to that of the s.c. tumors (Fig. 4). In the pulmonary parenchyma there are solid masses of tumor cells, some growing inside some of the larger blood vessels. Fig. 5 shows a typical pulmonary tumor in lung parenchyma adjacent to a bronchus.

For characterization of the transplantability of the cells in the lungs, various numbers of cells were injected i.v. into male BD2F1 mice. Chart 2 shows the average number of lung tumor colonies/mouse when $1 \times 10^5$, $3 \times 10^5$, and $5 \times 10^5$ cells/mouse were injected. Each point on the chart represents the average of counts on 5 mice. The number of colonies increased with the increased number of injected cells. With the lowest inoculum, $1 \times 10^5$ cells/mouse, 3 of 5 mice did not develop visible foci during this time period (3 weeks). With $5 \times 10^5$ cells, about 50 colonies/mouse were counted, but mice with over 50 colonies/mouse could not be counted accurately and are recorded as having 50 colonies. Plating efficiency of the cells was determined by seeding 50 cells/4 ml of medium in Falcon plates and incubating for 10 days. The colonies were stained with Wright's stain as described previously. The average number of colonies in 6 plates was $6.2 \pm 0.6$ plate.

Chart 3 shows the effect of injecting microspheres with the cells upon lung colony production. Male BD2F1 mice were each inoculated with $2 \times 10^6$ cells plus varying numbers of microspheres. Each value represents the average of counts on 5 mice. Microspheres showed some enhancement of lung colony formation in this range.

Table 1 shows the average numbers of lung tumor colonies/mouse when $1 \times 10^5$, $3 \times 10^5$, and $5 \times 10^5$ cells/mouse were inoculated with $5 \times 10^5$ microspheres. The result in

<table>
<thead>
<tr>
<th>No. of cells ($\times 10^5$)</th>
<th>No. of colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.2 $\pm$ 1.7$^a$</td>
</tr>
<tr>
<td>3</td>
<td>24.4 $\pm$ 6.7</td>
</tr>
<tr>
<td>5</td>
<td>47.4 $\pm$ 2.6</td>
</tr>
</tbody>
</table>

$^a$ Mean $\pm$ S.E.
this experiment was very similar to that of Chart 2, although results in Table 1 included the addition of microspheres.

As a preliminary test of this system for sensitivity to antimetabolites, ara-6MP and SeGR were used to treat KLN205 cells in culture (Chart 4). Flasks were inoculated with $2 \times 10^5$ cells each in 10 ml medium, and ara-6MP or SeGR was added at $5 \times 10^{-8}$ M. Duplicate flasks containing cells treated with each drug were taken out and counted every second day, and growth was compared with control flasks. This concentration of SeGR completely inhibited cell growth (6-selenoguanine has since been found to be equally effective). The ara-6MP had an effect only after 4 days of incubation. The response of KLN205 cells in vivo was also determined. Groups of 5 male BD2F₁ mice were each given injections of $3 \times 10^6$ cells plus $5 \times 10^5$ microspheres/mouse. The mice were treated by i.p. injection of 0.9% NaCl solution or drug twice daily for 7 days, beginning 1 day after transplantation of the cells. The doses were 16.6 mg/kg for SeGR and 10 mg/kg for ara-6MP. At 3 weeks the mice were sacrificed, and lung colony counts were performed. The results are shown in Chart 5. The SeGR treatment markedly reduced lung colony formation, but the effect of treatment with ara-6MP was marginal. SeGR treatment started 4 days after transplantation showed very similar inhibitory effects on lung colony formation. Since the data were so similar, they were omitted. This probably indicates direct antitumor effects rather than effects upon vascularization.

While presently available drugs have limited effects on human lung cancers, some information concerning the responses of this model system to those drugs that have some clinical utility would be of interest. Table 2 shows tests of 6 examples of such drugs tested in vitro against growth of KLN205 cells. 5-Fluorouracil, thioguanine, methotrexate, and Adriamycin had some inhibitory effects. Methylcyclohexyl-nitrosourea had no effect at this dose. Table 3 shows the results of similar tests in vivo with the same drugs and with Cytoxan. In this case there were significant effects only with thioguanine and hexamethylmelamine.

**DISCUSSION**

The cloned line of this squamous cell carcinoma of the inbred DBA/2 mouse line grows reproducibly in cultures and reproducibly causes lung tumor growths when injected as suspensions by tail vein. It is useful to be able to test the same tumor line in vitro, where cytotoxicity can be monitored with small amounts of new agents, and then extend study of positive tests to the in vivo model. SeGR produced parallel responses in vitro and in vivo. ara-6MP was tested in vitro at $5 \times 10^{-8}$ M, a concentration known from earlier studies to be effective in inhibiting cytidylate reductase in responsive tumor cells (1-3). A response was seen only after 4 days of incubation. This may mean that the FCS component of the medium supplied sufficient DNA precursors to the cells for this time period. The dose of ara-6MP used in vivo (10 mg/kg) would produce equivalent concentrations in body water, but renal clearance is rapid (1-3).

Table 2

<table>
<thead>
<tr>
<th>Drugs used</th>
<th>No. of cells per flask</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-(2-chloroethyl)-3-(trans-4-methyl-cyclohexyl)-1-nitrosourea</td>
<td>$24.1 \times 10^8$</td>
</tr>
<tr>
<td>Adriamycin</td>
<td>$1.6 \times 10^4$</td>
</tr>
<tr>
<td>Hexamethylmelamine</td>
<td>$23.1 \times 10^4$</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>$7.31 \times 10^4$</td>
</tr>
<tr>
<td>Thioguanine</td>
<td>$1.57 \times 10^4$</td>
</tr>
<tr>
<td>5-Fluorouracil</td>
<td>$4.26 \times 10^4$</td>
</tr>
<tr>
<td>Control</td>
<td>$25.5 \times 10^4$</td>
</tr>
</tbody>
</table>
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Table 3
Effects of several drugs on the formation of lung colonies by KLN205 cells

Male BD2F1 mice were inoculated with 3 × 10⁶ cells and 5 × 10⁶ microspheres per mouse. They were treated as indicated by i.p. injection starting 4 days after inoculation. Mice were sacrificed 3 weeks after transplantation, and the lung colonies were counted. Each value is an average of counts on 10 mice. The p values determined by Student’s t test are: hexamethylmelamine treated, p < 0.001; and thiopurine treated, p < 0.001 (as compared with control). The results with the other agents were not significantly different from the controls.

<table>
<thead>
<tr>
<th>Drugs used</th>
<th>Treatment</th>
<th>Av. no. of colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-[2-chloroethyl]-3-(trans-4-methyl-cyclohexyl)-1-nitrosourea</td>
<td>24 mg/kg, 1 time daily, 1 day</td>
<td>20.8 ± 3.7⁷</td>
</tr>
<tr>
<td>Adriamycin</td>
<td>2 mg/kg, 1 time daily, 5 days</td>
<td>19.1 ± 5.9</td>
</tr>
<tr>
<td>Cytoxan</td>
<td>15 mg/kg, 1 time daily, 5 days</td>
<td>23.3 ± 4.8</td>
</tr>
<tr>
<td>Hexamethylmelamine</td>
<td>20 mg/kg, 1 time daily, 5 days</td>
<td>4.5 ± 1.6</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>1.5 mg/kg, 1 time daily, 5 days</td>
<td>23.5 ± 4.8</td>
</tr>
<tr>
<td>Thioguanine</td>
<td>4 mg/kg, 1 time daily, 5 days</td>
<td>5.9 ± 1.8</td>
</tr>
<tr>
<td>5-Fluorouracil</td>
<td>20 mg/kg, 1 time daily, 5 days</td>
<td>29.7 ± 5.2</td>
</tr>
<tr>
<td>Control</td>
<td>0.9% NaCl solution, 0.1 ml, 1 time daily, 5 days</td>
<td>26.4 ± 4.3</td>
</tr>
</tbody>
</table>

⁷ Mean ± S.E.

and more frequent treatment may be required. The doses used earlier by Kimball et al. (1-3) were 60 to 100 mg/kg twice daily.

The model system appears to be responsive to several appropriate drugs in terms of cytotoxicity to cells in culture. However, as might be expected, the responsiveness is much less extensive in vivo. The responsiveness to SeGR thus appears more significant.

ACKNOWLEDGMENTS

The authors are indebted to Dr. P. Nettesheim for supplying DBA/2 mice bearing the lung carcinoma. They also wish to acknowledge the advice and assistance of colleagues, Dr. C. E. Cass and Dr. A. R. P. Paterson in the cell culture work and of Dr. G. O. Bain, Department of Pathology, University of Alberta, for the histological examinations of tumor nodules.

REFERENCES

Fig. 1. A, KLN205 cells grown *in vitro*. Wright’s stain, × 160. B, KLN205 cells grown *in vitro*. Wright’s stain, × 400.

Fig. 2. The histology of s.c. tumor produced by inoculation of KLN205 cells into male DBA/2 mice. H & E, × 300.

Fig. 3. A typical lung preparation with tumor nodules produced by i.v. inoculation of KLN205 cells into male BD2F mice.
Fig. 4. The histology of a lung tumor nodule produced by i.v. inoculation of KLN205 cells into male BD2F1 mice. H & E, × 300.

Fig. 5. The histology of a lung tumor nodule produced by i.v. inoculation of KLN205 cells into male BD2F1 mice, showing the margin. H & E, × 120.
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