ABSTRACT

When mixtures of cell lines 168 and 4T07, both derived from the same mouse mammary tumor, were injected into syngeneic mice, the resulting tumors, analyzed over a large size range by colony-forming assays in selective media, consisted primarily of line 4T07, even when the ratio injected was 100:1 or greater in favor of line 168. This result indicated a suppression of growth of line 168, since the volume-doubling time of line 168 tumors in the absence of line 4T07 was one-half that of line 4T07 tumors. That growth suppression was not due to inhibition of line 168 by immunity induced to line 4T07 was shown in two ways: (a) line 168 tumors grew almost as well in mice preimmunized with line 4T07 as in controls, whereas line 4T07 tumor growth was strongly inhibited in preimmunized mice; and (b) the final composition (favoring line 4T07) in mixed tumors was similar in tumors grown in mice immunosuppressed by irradiation to that in nonirradiated controls. The strong suppression of line 168 did not occur when the two cell lines were injected simultaneously at different s.c. sites, nor did it occur when line 168 cells were injected in mixtures with lethally irradiated line 4T07 cells.

Line 4T07 cells also suppressed the growth of line 168 cells in monolayer cultures. It was not likely that suppression was due to competition for growth factors, since the effect required cell contact. Suppression probably was not mediated through junctional communication, since these cells do not engage in metabolic cooperation. We suggest that a growth-inhibitory factor produced by line 4T07 mediates the suppression of 168 cells.

INTRODUCTION

It is now widely accepted that multiple subpopulations of tumor cells which differ in a number of heritable characteristics can be isolated from single tumors (for reviews, see Refs. 1–5). We have been working with such a system, consisting of a series of subpopulation lines derived from a mammary tumor which arose in a BALB/cfC3H mouse (6, 7). These subpopulations differ in many characteristics, including growth properties (8–10), propensity to metastasize (11), and intrinsic sensitivity to antineoplastic drugs (7, 12–15). These differences are evident when the subpopulations are tested individually. In order to determine the extent to which subpopulations influence and interact with each other when they are grown in each other’s presence, we have studied the behavior of paired mixtures of subpopulation lines, both in vivo and in vitro.

As we have described previously (16), when mixtures of two of these lines, line 168 and line 4T07, are injected s.c. into syngeneic mice, the resulting tumors consist primarily of line 4T07 cells and host cells; few line 168 cells are found. In many tumors we analyzed, less than 0.5% (the limit of detection) of the tumor cells were line 168, even when we injected up to 19 times as many line 168 cells as 4T07. This dominance of line 4T07 over line 168 was unexpected, since line 168 formed fast-growing tumors in the absence of line 4T07. Our previous studies (8) with line 168 and line 410, a line from which line 4T07 was indirectly derived, had shown that when these two tumor lines are injected at different s.c. sites on the same mouse, the growth of line 168 is slowed or suppressed by the presence of line 410 (as is the growth of a second line 410 tumor). We showed that this suppressive effect of line 410 on line 168 is due to an immune response to line 410 which can retard the growth of both tumors, even though line 168 is nonimmunogenic (8). We report here experiments designed to determine whether the dominance of line 4T07 over line 168 is also effected through an immune response or whether some other mechanism or mechanisms are at work.

MATERIALS AND METHODS

Cell Lines. Cell line 168 and 410.4 were isolated from a single, spontaneously arising mammary tumor of a BALB/cfC3H mouse (6, 17). A thioguanine-resistant, ouabain-resistant cell line, 44FT0, was isolated from line 410.4 after mutagenesis with ethyl methanesulfonate (18). Line 4T07 was isolated from line 44FT0 after seven in vivo passages through the lungs of syngeneic mice (16). Both cell lines were certified *Mycoplasma* free, using DNA fluorochrome stain plus UV microscopy, by Bionique Laboratories (Saranac Lake, NY). Both cell lines are nonmetastatic in a s.c. site, although both form lung colonies after i.v. injection.

Media. For routine cell culture, cells were grown in DME−10 medium, consisting of DME supplemented with mixed nonessential amino acids (1 mm), 2 mm L-glutamine, penicillin (100 units/ml), streptomycin (100 μg/ml), 5% iron-supplemented calf serum (low endotoxin serum, Hyclone Sterile Systems, Inc., Logan, UT), and 5% fetal bovine serum (low endotoxin serum, Grand Island Biological Co., Grand Island, NY). For blastogenesis assays, SDME-10 medium was used: DME-10 was supplemented further with 20 μM glucose, 8 μM bovine insulin, 1 mM oxaloacetic acid, and 10% NCTC 109 medium (Microbiological Associates Bioproducts, Walkersville, MD). Fetal bovine serum was substituted for calf serum in this medium. DME medium in powder form and other culture supplements were from Grand Island Biological Co.

Mice. Male BALB/c mice, 8 to 13 weeks old, were produced in our animal colony, from a BALB/c breeding colony established by cesarean derivation of a litter of mice from BALB/cfC3H parents obtained from the Cancer Research Laboratory, Berkeley, CA.

Tumors. Cells from monolayer culture were suspended in Hanks’ buffered salt solution and injected s.c. into mice in a volume of 0.1 ml. Tumors were measured twice a week in two perpendicular dimensions with vernier calipers. Mice were sacrificed and their tumors were aseptically removed for cell suspension when tumors reached a size of 90–3000 mm³. Tumor size in mm³ was calculated by the formula $a - b^2$, where $b$ is the smaller and $a$ the larger of the two tumor dimensions.

For each individual tumor, the tumor volume between approximately 75 and 500 mm³ was fitted to an exponential growth curve, using linear regression analysis of the logarithm transformation of tumor volume. Values for time to reach 100 mm³ and tumor-doubling time were obtained from each fitted curve.

Tumor Cell Suspension. Tumors were cut into pieces with scalpels, digested for 1 h with 2 mg/ml collagenase, type 3 (Cooper Biomedical, Malvern, PA), and 1 mg/ml hyaluronidase (Sigma Chemical Co., St. Louis, MO), as described previously (16). Our previously published
procedure was modified in that the protease step was omitted. After collagenase digestion, cells were rinsed, passed through a syringe, and suspended as described previously (16). A portion of each cell suspension was diluted and counted with trypan blue to determine the percentage of live cells.

Identification of Tumor Subpopulations in Mixtures by Colony Formation Assay. The description and validation of this method have been published previously (16). Briefly, cells were diluted and plated at 5000, 1000, and 200 live (trypan blue-excluding) cells/well in 6-well tissue culture plates containing an equal volume of selective medium 2-fold concentrated in selective agent. Selective media used were thioguanine medium (DME-10 containing 60 μM 6-thioguanine) for line 4T07 and hypoxanthine-aminopterin-thymidine medium (DME-10 containing 100 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine) for line 168. Thioguanine and hypoxanthine-aminopterin-thymidine mixtures were obtained from Sigma. After 7 to 10 days incubation at 37°C in a 10% CO₂/air atmosphere, colonies were fixed in methanol:acetic acid (2:1), stained with crystal violet, and counted with the aid of a dissecting microscope. The percentage of each tumor line present in tumor cell suspensions was calculated by a colony-forming efficiencies in the two media as described previously (16).

Splenocyte Preparation. Mice were sacrificed by cervical dislocation, spleens were removed and weighed, and splenocytes were prepared by mechanical disruption and RBC lysis as described previously (16).

Blastogenesis Assays. Spleen cells were suspended at 5 × 10⁶ cells/ml in SDME-10, and dispensed at 0.1 ml/well in 96 well round bottomed plates. To each well was added 0.1 ml SDME-10 containing either concanavalin A (Calbiochem Biochemicals, San Diego, CA) or lipopolysaccharide W (Difco Laboratories, Detroit, MI) to the final concentration noted in “Results.” Three to 6 replicate wells were set up for each drug concentration, as well as six to 12 controls without drug. After 48 h incubation at 37°C in a 10% CO₂/air atmosphere, 1 μCi of [³H]thymidine was added in 50 μl SDME-10/well. After 18 h of further incubation, cells were harvested with a MASH II Multiple Automated Sample Harvester (Microbiological Associates), the filter paper disks containing cells were placed in counting vials with scintillation fluid, and the amount of radioactivity was measured by a liquid scintillation counter.

Irradiation for Immunosuppression. Mice were treated with 450 rads whole body irradiation, administered by a 137Cs Gamma Irradiator (J. L. Sheperd and Assoc., Glendale, CA), at 106 rads/min, 2 days prior to tumor cell injection.

Surgical Removal of Tumors. Mice were anesthetized with 65 mg/kg sodium pentobarbital for surgery. Subcutaneous tumors were removed, and wound clips were used to close the skin. In control mice without tumors, a large portion of the No. 4 mammary fat pad was removed in a similar surgery.

Collagen Gel Cultures. This 3-dimensional culture system has been described previously (13, 14). Briefly, cells from monolayer cultures were suspended with trypsin, centrifuged to form a pellet, and resuspended at 10⁴ to 2 × 10⁶ cells/μl of complete collagen mixture. One μl per well was embedded in individual wells of 24-well plates containing 0.5 ml complete collagen mixture and then overlaid first with 0.4 ml complete collagen mixture and then with 0.9 ml DME-10. The medium above the gel was changed twice a week. Cells were removed from collagen for colony formation assays as follows. The collagen gel containing the cell bolus was removed from the well with a Pasteur pipet and placed in a centrifuge tube. One ml containing 2 mg/ml collagenase, type 3 was added to each tube, and tubes were incubated in a shaking water bath at 37°C. After 1 h, 10 ml medium were added to each tube, and tubes were centrifuged to pellet cells. The cell pellets were rinsed with a second 10 ml medium, resuspended, pipetted up and down to break up clumps, and passed 4 times through a syringe with a 25–27-gauge needle to form a single cell suspension.

RESULTS

Growth of 4T07 Cells and 168 Cells in Mixtures in Vivo. We have repeated our experiments (16) with 168:4T07 mixtures at increasingly higher injection ratios of line 168 to 4T07 (Table 1). Clearly, line 4T07 strongly dominates line 168 in tumors arising from mixtures of the two. This dominance was not predictable, since line 4T07 alone forms slower growing tumors than does line 168 alone. As shown in Table 2, in five separate experiments, although line 4T07 tumors are significantly quicker to reach a size of 100 mm³, they have a significantly slower volume-doubling time during exponential growth (determined between 75 and 500 mm³). In addition, line 168 tumors tend to continue to grow exponentially to larger sizes (up to 2000 mm³), whereas line 4T07 tumors often slow in growth after they reach a size of 500 to 800 mm³ (not shown). These data indicate that if lines 168 and 4T07 grew independently in each other’s presence, line 168 would be expected to dominate in larger tumors. However, when we tested tumors over a larger size range, the largest tumors did not contain a significantly higher proportion of line 168 cells than did the smallest (Table 3). These data clearly indicate that the two cell lines do not grow independently of each other in tumors arising in mixtures.

Because of our previous experiments investigating the effect on line 168 of line 410, an indirect ancestor to line 4T07 (8), we suspected that all or part of the suppressive effect of line 4T07 on line 168 was due to an immune response of the host, triggered by line 4T07, to which line 168 was responsive. However, we found little evidence that such an immune response played a role in the 168-4T07 interaction.

When we immunized mice to line 4T07 and surgically removed the tumors which arose, 15 of 16 mice challenged with

| Table 1 Line 4T07 dominance in tumors arising from mixtures of lines 168 and 4T07 |
|---------------------------------|-----------------|
| 168:4T07 ratio injected | % of tumor cells of each tumor which were line 168 |
| 30:1 | 6 ± 9 (14)° |
| 100:1 | 9 ± 11 (10) |
| 1000:1 | 58 ± 18 (10) |

* Mean ± SD (number of tumors).

| Table 2 Growth characteristics of 168 and 4T07 tumors |
|---------------------------------|-----------------|
| Tumor cells were injected s.c. at 3 × 10⁶ cells/mouse. |
| Tumor line | Days to reach 100 mm³ | Volume-doubling time |
|---------------------------------|-----------------|
| 168 | | |
| 1 | 26 ± 4 (9)* | 2.9 ± 0.8 |
| 2 | 23 ± 3 (8) | 3.0 ± 0.7 |
| 3 | 20 ± 3 (10) | 3.3 ± 1.0 |
| 4 | 17 ± 2 (9) | 2.3 ± 0.8 |
| 5 | 21 ± 5 (10) | 2.2 ± 0.4 |
| Mean | 21 | 2.7 |
| 4T07 | | |
| 1 | 15 ± 7 (10) | 6.0 ± 2.1 |
| 2 | 14 ± 5 (10) | 6.4 ± 2.3 |
| 3 | 14 ± 4 (7) | 5.9 ± 1.4 |
| 4 | 13 ± 3 (10) | 6.7 ± 1.3 |
| 5 | 18 ± 6 (10) | 4.0 ± 1.5 |
| Mean | 15 | 6.0 |

* Mean ± SD (number of tumors).

| Table 3 Tumor cell content of tumors of various sizes arising from 168-4T07 mixtures |
|---------------------------------|-----------------|
| Tumor cells were injected s.c. at 3 × 10⁶ total cells at a 3:1 168:4T07 ratio and harvested at the sizes shown. Each tumor was analyzed separately for subpopulation content. |
| Tumor size at harvest (mm³) | No. of tumors analyzed | % of tumor cells in each tumor which were line 168 |
|---------------------------------|-----------------|
| Small: 88–220 | 9 | 2.6 ± 1.7° |
| Medium: 350–450 | 5 | 1.7 ± 0.6 |
| Large: 650–2750 | 6 | 1.7 ± 2.0 |

* Mean ± SD.

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10^5 line 168 tumor cells developed tumors, as did 10 of 10 control (nonimmunized) mice and 7 of 8 mice preimmunized with line 168 tumors (Table 4). As shown in Table 4, line 168 tumors injected into 4T07-immunized mice were only slightly delayed in time to reach 100 mm^3 (difference of means, 4 days; difference of medians, 7 days). By contrast, line 4T07 tumors injected into 4T07-immunized mice were strongly delayed in time to reach 100 mm^3 (difference of medians, 37 days). Volume-doubling time was not affected by preimmunization in any group (not shown). Clearly, line 4T07 immunizes more strongly against itself than against line 168. The degree of immunity against line 168 is insufficient to explain the strong growth suppression by line 4T07 in mixed tumors.

In our previous work with line 168 plus line 410 tumors, immunosuppression of the host abrogated the ability of line 410 to interfere with the growth of line 168 (8). By contrast, when line 168-4T07 mixtures were injected into mice immunosuppressed by irradiation (450 rads γ-irradiation 2 days prior to cell injection), little abrogation of the inhibitory effect of 4T07 on 168 was seen (Fig. 1). Most tumors which arose from 1:1 and 5:1 ratios of injected cells contained few line 168 cells at the time of tumor harvest in irradiated mice as well as in controls.

That the host mice were immunosuppressed by the irradiation procedure was confirmed both by decrease in spleen weights and number of splenocytes recovered after irradiation and by functional assays for splenocytes. Splenocyte number from 4 nonirradiated control mice averaged [72 ± 21 (SD)] × 10^6 cells after RBC lysis; this value dropped to [7 ± 4] × 10^6 2 days after irradiation, gradually increasing thereafter. As shown in Fig. 2, blastogenesis assays using either a T-cell mitogen (concanavalin A) or a B-cell mitogen (lipopolysaccharide) confirmed that splenocyte function was severely depressed on day 2 after irradiation and recovered thereafter but was not entirely recovered even by day 10 after irradiation (the last day assayed). Likewise, the number of splenocytes had not totally recovered by day 10, averaging (28 ± 16) × 10^6 on that day.

Our previous experiments demonstrating 410-168 interaction in vivo were carried out by injecting line 168 cells at one s.c. site and line 410 cells at a second site. Using this procedure, we saw that injection of line 410 caused a decreased incidence or increased latency of either line 168 tumors or line 410 tumors injected opposite (8). In contrast, line 168 had no effect on either line 168 or line 410 tumors injected opposite (8).

Table 4: Growth of line 168 and 4T07 tumors in preimmunized mice

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Preimmunization</th>
<th>Challenge</th>
<th>Incidence</th>
<th>Days to reach 100 mm^3</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>168</td>
<td>10/10^6</td>
<td>24 ± 6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>168</td>
<td>10/10^6</td>
<td>25 ± 5</td>
<td>NS^d</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4T07</td>
<td>15/16</td>
<td>28 ± 4</td>
<td>NS^d, P &lt; 0.05^d</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>None</td>
<td>4T07</td>
<td>10/10</td>
<td>19 ± 8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4T07</td>
<td>7/8</td>
<td>22 ± 9</td>
<td>NS^d</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4T07</td>
<td>6/8</td>
<td>42 ± 17</td>
<td>P &lt; 0.01^d, P &lt; 0.002^d</td>
<td></td>
</tr>
</tbody>
</table>

^a Number of mice with tumors/number of mice given injections.
^b Mean ± SD of tumors which developed.
^c Significance tested by t test, compared to control (not preimmunized) group. Only mice which developed tumors were included. NS, not significant.
^d Significance tested by Wilcoxon two-sample test, compared to control group. All mice were included; mice which did not develop tumors were assigned values of >65 days (when experiment was terminated).

When we repeated this procedure with line 168 and line 4T07 cells, a small yet significant delay in the growth of line 168 cells with 4T07 opposite was seen, both in the time to reach 100 mm^3 and in the doubling time (Table 5). However, a similar (although not statistically significant) delay in time to reach 100 mm^3 was also seen in the growth of 4T07 tumors opposite line 168 tumors and in the growth of 4T07 tumors opposite 4T07 tumors (Table 5).

Suppression of the growth of line 168 by 4T07 required replicating line 4T07 cells. Injection of 3 × 10^6 lethally irradiated 4T07 cells with 3 × 10^5 viable 168 cells caused only a slight delay in the growth of line 168 cells (Table 6). This is further evidence that the strong suppression of line 168 by nonirradiated line 4T07 does not occur because of immunity induced to line 4T07.

Growth of 4T07 Cells and 168 Cells in Mixtures in Vitro. Since there was so little evidence for immunity as the mechanism through which line 4T07 suppresses the growth of line 168, we attempted to discover whether line 4T07 cells could suppress the growth of line 168 cells in vitro. As shown in Fig. 3, when mixtures of these cells were plated in monolayer, line 4T07 cells overgrew line 168 cells after several passages. In parallel unmixed cultures, the doubling time of line 4T07 averaged 16 h, whereas the doubling time of line 168 averaged 13.5 h, so this overgrowth could not be explained on the basis of the better growth of 4T07 in culture. Plating efficiencies of both lines averaged 50%. In the experiment shown in Fig. 3, cells were plated heavily, at 10^4 cells/well of 6-well plates (10^6 cells/cm^2), and cultures reached confluency before replating. In parallel wells in the same experiment, in which cells were plated at 10^4 cells/well, mixed cultures retained approximately equal numbers of line 168 and 4T07 through 4 passages (not shown), suggesting that line 4T07 asserts its dominance only when there is a deficiency in available resources or space. As shown in Fig. 3, the inhibitory effect of line 4T07 appeared to be on the growth of line 168 rather than on plating efficiency: cultures analyzed 5 h after plating had about the same proportions as
DOMINANCE IN TUMOR CELL POPULATIONS

Fig. 2. Blastogenesis assays with splenocytes from control versus irradiated mice. Mice were irradiated as described in Fig. 1. Individual mice were randomly selected, and splenocytes were prepared and assayed as described in "Materials and Methods" at various days after irradiation: V, 2 days; O, 4 days; •, 7 days; O, 10 days; •, nonirradiated controls. For irradiated animals, each point is the mean of 4 animals (6 to 12 replicate wells/animal); hnr. SE.

Table 5 Growth of line 168 and 4T07 tumors either alone or with another tumor at a second s.c. site

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Tumor</th>
<th>Time to reach 100 mm³ (days)</th>
<th>Tumor-doubling time (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>168 alone</td>
<td>23 ± 5 (10)</td>
<td>5.0 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>168 with 4T07</td>
<td>9 ± 5 (10)</td>
<td>5.0 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>4T07 alone</td>
<td>14 ± 5 (10)</td>
<td>6.4 ± 2.4</td>
</tr>
<tr>
<td></td>
<td>4T07 with 168</td>
<td>19 ± 5 (10)</td>
<td>5.5 ± 1.8</td>
</tr>
<tr>
<td>2</td>
<td>4T07 alone</td>
<td>14 ± 5 (10)</td>
<td>5.9 ± 3.1</td>
</tr>
<tr>
<td></td>
<td>4T07 with 4T07</td>
<td>16 ± 6 (17)</td>
<td>6.2 ± 2.2</td>
</tr>
</tbody>
</table>

* Mean ± SD (number of tumors).
* Significantly different from tumor grown alone, P < 0.01 by t test.
* Not significantly different from tumor grown alone.

Table 6 Effect of lethally irradiated 4T07 cells on growth in vivo of line 168 tumors

<table>
<thead>
<tr>
<th>Cell injected</th>
<th>Time to reach 100 mm³ (days)</th>
<th>Volume-doubling time (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Line 168 (3 x 10⁶/mouse)</td>
<td>18 ± 2 (9)</td>
<td>2.2 ± 1.0</td>
</tr>
<tr>
<td>Line 168 (3 x 10⁶/mouse) plus</td>
<td>21 ± 3 (9)</td>
<td>2.9 ± 0.6</td>
</tr>
<tr>
<td>4T07 (3 x 10⁶/mouse)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Mean ± SD (number of animals).
* Significantly different from controls, P < 0.02 by t test.
* Not significantly different from controls.

The inhibitory effect of line 4T07 on line 168 in culture was confirmed in an experiment in which different ratios of the two lines were cocultured through 17 successive passages (Fig. 4). When 1:1 and 1:9 168:4T07 ratios were plated, the inhibition appeared during the second passage. However, when a 9:1 ratio was plated, line 168 at first continued to dominate the mixture, reaching 99% after the second passage. During the next two passages, however, the ratio dropped as line 4T07 cells began to overtake the culture; within 31 days (12 more passages), line 168 had dropped below the limit of detection.

We tested conditioned medium from confluent or semiconfluent 4T07 monolayer cultures for its ability to inhibit growth of line 168 cultures. Media from the line 4T07 cultures did inhibit growth of line 168, but no more effectively than did line 168-conditioned media; conditioned medium from mixed 4T07/168 cultures also inhibited growth of line 168 to a similar extent (not shown). Different batches of conditioned media varied widely in their ability to inhibit the growth of line 168; in 3 experiments, the growth of line 168 cells in 100% conditioned medium ranged from 0.3 to 57% of controls in fresh medium. This variation appeared to be correlated with the degree of confluency of the cultures when media were harvested. Conditioned medium from cultures in exponential growth had little effect on growth (not shown). The inhibitory effect of
operation (19), as revealed by a decrease in the number of
We have used this assay previously to measure metabolic co-
200 4T07 cells/dish in 60-mm dishes alone or with 1 x 10^5-7
had the ability to engage in metabolic cooperation. We plated
order to inhibit its growth, we tested whether these cell lines
both cell lines reached confluency before each repassage.
by the presence of line 4T07 over several passages, although
x IO5 line 168 cells, in medium containing 60 //M thioguanine.
in Table 7, growth of line 168 was not significantly inhibited
are very low, so the entire plate may be flooded with medium
(England). In this plate, individual well side walls
plating both cell lines in separate wells of a Sterilin 4-well plate
of line 168 in monolayer when cells were not in contact, by
factor. We tested the ability of line 4T07 to inhibit the growth
conditioned medium from confluent cultures is unlikely to be
related to the inhibition of line 168 by line 4T07 in vitro, since
there was no specificity.
Our inability to detect a specific inhibitory effect with con-
tioned media, as well as the effect of density on inhibition
(see above), suggested that cell-cell proximity was an important
factor. We tested the ability of line 4T07 to inhibit the growth
of line 168 in monolayer when cells were not in contact, by
plating both cell lines in separate wells of a Sterilin 4-well plate
(Middlesex, England). In this plate, individual well side walls
are very low, so the entire plate may be flooded with medium
while cells plated in individual wells remain separate. As shown
in Table 7, growth of line 168 was not significantly inhibited
by the presence of line 4T07 over several passages, although
both cell lines reached confluency before each repassage.
Because line 4T07 seemed to require contact with line 168 in
order to inhibit its growth, we tested whether these cell lines
had the ability to engage in metabolic cooperation. We plated
200 4T07 cells/dish in 60-mm dishes alone or with 1 x 10^5-7
x 10^5 line 168 cells, in medium containing 60 //M thioguanine.
We have used this assay previously to measure metabolic co-
operation (19), as revealed by a decrease in the number of
colonies able to grow in thioguanine (line 4T07) in the presence
of increasing numbers of thioguanine-sensitive communicating
cells (line 168). No difference in colony numbers were found
over the entire range of 168 cells added, suggesting that meta-
boic cooperation does not occur between these cell lines (not shown).
We also tested the ability of line 4T07 to dominate line 168
in mixed cultures grown in collagen gels. In these cultures, a
bolus of cells is embedded between two layers of collagen gel,
overlaid with medium, and allowed to grow for 7 to 14 days
with periodic changes of medium. Bolus size (cross-sectional
area) can be measured nondestructively during bolus growth.
Periodically, individual boluses can be harvested, and their cells
can be isolated, counted, and identified by colony-forming
assays in selective media. Results of one such experiment with
lines 168, 4T07, and their mixture are shown in Fig. 5. As
shown, line 4T07 grew poorly in collagen in comparison to line
168 in terms of net increase in cell number, even though the
size of 4T07 boluses increases rapidly in comparison with line
168 and most other cell lines (not shown). That line 4T07 in
collagen increases slowly or not at all in cell number has been
confirmed in other experiments. This characteristic is also
expressed by its indirect ancestor, line 410 (14). In boluses
formed from mixtures of lines 168 and 4T07, line 168 came to
dominate the boluses (Fig. 5). This result was confirmed in two
other experiments (not shown). Thus, when line 4T07 is placed
in culture under conditions in which its growth is arrested, it
does not inhibit the growth of line 168.

Table 7  Effect of coculture without cell contact on line 168 growth in monolayer

<table>
<thead>
<tr>
<th>Cell opposite</th>
<th>Passage</th>
<th>Growth of 168 cells (% of control)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>4T07</td>
<td>1</td>
<td>121 ± 13*</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>84 ± 16</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>99 ± 25</td>
</tr>
</tbody>
</table>

*a Within each experiment the number of 168 cells (plated at 3 x 10^5) at the
time of passage in wells with line 168 opposite was compared to the cell number
in wells with line 4T07 opposite.
*b The mean ± SD of 3 experiments.

conditioned medium from confluent cultures is unlikely to be
related to the inhibition of line 168 by line 4T07 in vitro, since
there was no specificity.

Fig. 4. In vitro growth of mixtures of 168 and 4T07 mixed at different ratios. Both lines alone, and 1:1 (C), 1:9 (Δ), and 9:1 (O) 168:4T07 mixtures were plated at 2.5 x 10^5 cells/T-25 flask in DME-10. Cells were replated twice a week for 15 to 17 passages. At the time of passages 1, 3, 5, 7, 15, and 17, some cells from each culture were plated in selective media for colony formation.

Fig. 5. Growth of lines 168, 4T07, and their mixtures in collagen gel cultures. Both cell lines alone and a 1:5 168:4T07 mixture were embedded in collagen gel at day 0. Two boluses of each type were harvested at various times as shown, and assayed for colony formation in selective media. Bottom, number of colony-forming cells per bolus. Top, percentage of colony-forming cells in mixed boluses found to be line 168 (mean of 2 at each time point), calculated from values shown in lower panel.

Day After Embedding

Growth of lines 168, 4T07, and their mixtures in collagen gel cultures. Both cell lines alone and a 1:5 168:4T07 mixture were embedded in collagen gel at day 0. Two boluses of each type were harvested at various times as shown, and assayed for colony formation in selective media. Bottom, number of colony-forming cells per bolus. Top, percentage of colony-forming cells in mixed boluses found to be line 168 (mean of 2 at each time point), calculated from values shown in lower panel.
DISCUSSION

We have shown that line 4T07 can suppress the growth of line 168 in vivo and in vitro in monolayer. To do so, line 4T07 cells must not only be viable but able to replicate. Although we cannot be sure whether the same mechanism(s) are responsible for the suppression in vivo and in monolayer, we have clearly shown that the suppression does not occur through an immune response triggered by line 4T07.

Possible mechanisms by which line 4T07 could inhibit growth of line 168 include production of inhibitory factor(s) by line 4T07 or competition for serum-derived or autocrine/paracrine factors, nutrients, attachment factors, or space. Both in vivo and in monolayer, strong growth inhibition of line 168 by line 4T07 apparently requires cell contact or at least close apposition of cells. Because neither conditioned medium nor growth factors from serum is the mechanism for growth inhibition of line 168, we believe that it is unlikely that competition for nutrients or growth factors from serum is the mechanism for growth inhibition of line 168. The experiment of Fig. 4 indicates that a small number of 4T07 cells can inhibit the growth of a large number of 168 cells, in monolayer as well as in vivo. These data also suggest that strict competition for either nutrients, growth factors, attachment factors, or space is unlikely to be the mechanism by which inhibition is achieved.

The experiment of Fig. 3 indicates that line 4T07 is capable of inhibiting the growth of line 168 in mixtures when cells are plated at a relatively high density (10^6 cells/cm^2). At this plating density, the cells reached confluency before they were replated. The inhibition in this and other experiments always occurred after the first or later passage. It may be that some time together at confluent or near confluent density is necessary to trigger the growth inhibition of line 168.

We believe the most likely explanation for our results is that line 4T07 produces some factor(s) which inhibit the growth of line 168. It may be that the two cell types must be in close proximity or in contact to trigger the production or activation of the factor, which is apparently short-lived. Alternatively, the inhibition itself may require contact.

The lack of inhibition of line 168 by line 4T07 in cell boluses growing in collagen was very surprising to us, since some of our other studies have led us to believe that this in vivo model better approximates in vitro tumor growth than does monolayer culture (13, 14, 18). Cells growing in collagen cultures as compared to monolayer have fewer cells in cycle (14) and are in close contact; cells in collagen are embedded as 10^5 cells in a bolus only 1 to 2 mm in diameter, which appears to be only one or two cells thick at the time of embedding. This density is >100 times the usual plating density of cells in monolayer. Our in vivo data indicate that line 4T07 must grow in order to inhibit line 168; irradiated line 4T07 cells did not strongly inhibit line 168 in mixed tumors (Table 6). We speculate that a period of exponential growth of mixed cultures is required to trigger the expression of inhibitory factor(s) produced by line 4T07 in vitro as well as in vivo. Because of its poor growth, line 4T07 seems unable to dominate line 168 in collagen gel cultures.

A large number of growth-regulatory factors produced by normal and transformed cells have been identified and described in recent years. One of these, TGF-β, has some properties which suggest to us that the interaction between lines 4T07 and 168 we describe here may involve a similar factor. Many carcinoma cell lines are inhibited by TGF-β (20), whereas other carcinoma cell lines have lost the inhibitory response (20, 21). TGF-β released by cells in culture is often in an inactive form, presumably bound to a binding protein, requiring activation (22, 23). We plan to test further the hypothesis that a TGF-β-like factor produced by line 4T07 causes the inhibition of growth of line 168. If this is the case, it may be that close contact between the cell lines is necessary to cause the release or activation of the factor.

The results shown here demonstrate one possible outcome when two cell subpopulations of a heterogeneous tumor are mixed and injected: one subpopulation comes to dominate the other. Kerbel et al. (24) have also seen clonal dominance develop in both primary tumors and metastases when they inject heterogeneous populations of neo-transfected mouse mammary tumor cells s.c. into syngeneic hosts. However, as we have also demonstrated, there are other possible outcomes between mixtures of tumor subpopulation lines: we have seen the development of a stable mixture with one pair of cell lines and the tendency to form homogeneous tumors of either one or the other of the two cell lines with another pair (25). Leith et al. (26) have also demonstrated the formation of stable mixtures between human colon carcinoma lines growing in nude mice. Whether clonal dominance or balanced growth resulted, in every case described above, the composition of the tumors which arose from the injected cell mixtures was not predictable on the basis of the growth rates of the individual subpopulations.

We picture a tumor as a dynamic ecosystem, in which the various cell populations affect each other's growth and behavior, probably by a variety of mechanisms. Therapy has the potential to upset this dynamic balance, perhaps in multiple ways. Therapy which selectively eliminates some subpopulations may thereby change the balance among the remaining subpopulations; some forms of therapy may also directly affect some mechanisms of interaction among subpopulations. Characterization of the mediator(s) of interaction may be relevant both to understanding the population dynamics of tumors and to developing therapies aimed at upsetting mechanisms by which tumor subpopulations interact to the ultimate detriment of the host.

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REFERENCES


Dominance of a Tumor Subpopulation Line in Mixed Heterogeneous Mouse Mammary Tumors

Bonnie E. Miller, Fred R. Miller, David Wilburn, et al.


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