Immunological Properties of Malignant and Nonmalignant Sublines of L-Cells

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ABSTRACT

Selection for immunoresistance and malignancy of nonmalignant sublines of L-cells resulted in the procurement of malignant LS-cells which grew successfully and killed 70% of syngeneic C3H mice. Preliminary inoculation with L-cells protected 30% of the animals from subsequent challenge by LS-cells. The L- and LS-cells had the following immunological properties in common: (a) sensitivity to the complement lytic action; (b) the ability to absorb lymphocytes on their surfaces; (c) the ability to affect the immune reactions by metabolic products; and (d) sensitivity to cytotoxicity of macrophages. However, LS-cells appeared to be more resistant to lysis by alloimmune T-lymphocytes and were much more resistant to lysis by natural killer cells from Wistar rats and athymic nude mice. In syngeneic recipients, the immune response to L- and LS-cells, evaluated by the in vitro cytotoxicity test, was equal for both types of cells. Only a partial cross-reactivity between L- and LS-cells was noted in cellular and humoral immune reactions.

INTRODUCTION

Immunological characteristics of cells, namely, those which determine immunogenicity and immunosensitivity, appear in different combinations in various cells. For example, sensitivity to lysis by lymphoid cells (2, 16, 18, 24, 29), by complement (10, 33, 37), or by lymphotoxin (24); increased or decreased immunogenicity (9, 30); and the ability to suppress or enhance immune reactions through the release of immunologically active substances (28, 36, 38, 49) are known to be present in some types of cells and absent in others. Each of the above-mentioned characteristics is sure to affect the immunoresistance of tumor cells and should effect their malignancy. This parallels, to a certain extent, the principle of Foulds' tumor progression [independent combination of new characteristics acquired by the organism. The need for such an experimental approach became obvious in connection with research in which immunological control of the tumor growth process. The importance of such an approach is dictated also by the fact that the metastatic ability of tumors may be related to their immunological properties (1, 13, 21).

L-cells which were obtained in 1943 from fibroblast cultures of adult C3H mice (see Ref. 32) are represented now by a number of malignant and nonmalignant sublines (19, 48). In this work, we have selected a malignant LS subline from a nonmalignant subline of L-cells. We present data on a number of immunological characteristics of nonmalignant (L) and malignant (LS) sublines of L-cells. These data support the idea that the above-mentioned properties may appear in cells in different combinations and that some of them determine the growth of tumor cells in recipients. This report also deals with the characteristics of the cellular and humoral immune response to L- and LS-cells in syngeneic animals.

MATERIALS AND METHODS

Animals. Inbred male C3H, BALB/c, athymic nude mice of BALB/c background, and Wistar rats were used in this work. All animals were bred in the Laboratory of Experimental Animals of this Institute and were 2 to 3 months old when used.

Target Cells. The target cells used were: L and LS1 through LS7, different sublines of mouse L-cells; and MMT1, cells of a malignant line obtained from a mammary tumor of C3H/He mice. All cells were maintained in vitro in Eagle's medium supplemented with 10% bovine serum. Before the L and LS comparison study, continuous cell lines were treated with tetracycline; during the experimentation they were free of Mycoplasma. This was determined by monthly examination with fluorescent Hoechst 33258 stain (6) or by determining the 
[1H]uridine and [3H]uracil incorporation ratio (41).

Effector Cells. To evaluate the sensitivity of target cells to different immune reactions, the following effector cells were used: (a) immune lymphocytes obtained from BALB/c mice at the peak of cytotoxic activity (30); (b) peritoneal macrophages obtained from intact C3Hf mice 3 days after i.p. injection of 4% freeze-thawed starch gel; and (c) spleen cells from athymic nude mice and from Wistar rats which were used as natural killer cells. To investigate the cellular immune response in syngeneic animals, C3Hf mice received s.c. inoculation of 10⁶ L- or LS-cells in each leg. At various times after inoculation, lymph node and spleen cells from individual mice were obtained, and their cytotoxicity was assayed. In each experimental alternative, at least 5 immune and 5 control animals were tested. All effector cells were suspended in cold Hank's solution, washed twice, resuspended in Eagle's medium supplemented with 2% bovine serum, and then used in the cytotoxicity tests.

Humoral Immune Response and Absorption Test. C3Hf mice were immunized by a single s.c. inoculation of 1 to 2 x 10⁸ L- or LS-cells. After 30 days, the mice were bled by decapitation, and the sera from 4 to 6 animals were pooled. Four sera samples were tested for each alternative. A hyperimmune anti-L-serum was obtained by 4-fold s.c. or i.p. inoc-
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Population of 1- to 4 × 10⁸ cells/mouse at 20-day intervals. The sera were inactivated by heating for 30 min at 56°. In order to examine the antigenic characteristics of L-cells, the hyperimmune anti-L-serum was absorbed with different types of cells. One ml of serum was absorbed with 4 × 10⁸ lymphoid and with 10 × 10⁷ nonlymphoid cells, respectively.

The relative quantity of H-2 transplantation antigens on target cells was estimated by absorption of hyperimmune anti-H-2² serum from BALB/c mice. All absorptions were performed for 1 hr at room temperature and for all alternatives in triplicate. Absorbed sera were tested for residual antibodies by the complement-dependent cytotoxicity test.

Cytotoxicity Test. The cytotoxicity test with all types of effector cells was performed according to our modification of the method of Takasugi and Klein (26, 30). Briefly, a target cell suspension containing 8 to 10 × 10⁴ cells/ml in Eagle's medium supplemented with 10% bovine serum was seeded into special 0.6-mI microplate flasks. With this concentration of cells, 100 to 150 cells adhered to a surface of 0.8 sq mm at the bottom of each flask. The target cells were allowed to stand overnight at 37°. Subsequently, target cells were washed once with Eagle's medium, and effector cells suspended in Eagle's medium with 2% bovine serum were added to target cells. Three to 4 replicate flasks of each target-effector combination were incubated in 5% CO₂ for 18 to 48 hr at 37°. After incubation, the flasks were washed with warm Hank's solution, and the target cells remaining on the glass were fixed with formalin and stained with hematoxylin. The number of cells that survived was counted under an inverted microscope.

Cytotoxicity reactions with antisera and complement were performed in the same flasks which were used for the tests with lymphocytes. Three to 4 replicate flasks with target cells and each antisera dilution were incubated with fresh nontoxic rabbit complement (1:30 dilution) for 2 to 3 hr at 37°. The number of cells that survived indicated the sera and complement activity (29). In all experiments except those performed with syngeneic lymphocytes and with antiserum, the cytotoxicity index was used. This index was used in order to inhibit mitosis (26).

The mean values from each triplicate or quadruplicate experiment were used to calculate the percentage of lysis or the cytotoxicity index. The percentage of lysis was evaluated as [(a - b)/a] × 100, where a and b are the numbers of cells remaining after incubation in the control and experimental flasks, respectively. As a rule, each experiment was repeated at least 3 times. All the values were added, and the mean value and standard error were calculated. Differences between the mean values were analyzed by Student's t test, and in our assay more than 10% cytotoxicity was found to be consistently significant at p = 0.05. To express the cytotoxic potential of syngeneic effector cells, the cytotoxicity index was used. This was evaluated for each pair of control animals and immune mice as a/b, in which a and b indicate the number of cells after incubation with effectors from a control and an immune mouse, respectively. The values of matches were added and the mean ± S.D. was calculated.

Lymphocyte Absorption on Target Cells. The ability of L- and LS-cells to absorb lymphocytes on their surfaces was estimated as described previously (29). Briefly, target cells that adhered to the coverglass were incubated with lymphocytes in Eagle's medium supplemented with 2% bovine serum in a special chamber for 1 hr at 37°. After incubation, target cells were gently but thoroughly washed from nonadherent lymphocytes, fixed in ethanol, and stained with hematoxylin. The percentage of target cells with bound lymphocytes was counted under a microscope at ×900. No less than 12 preparations for each kind of target cell in 3 different experiments were examined. One thousand target cells were examined in each preparation.

Preparation of Conditioned Media. Conditioned media were obtained by incubation of 5 × 10⁶ proliferating cells in 10 ml of Eagle's medium supplemented with 2% bovine serum for 24 hr in Carrel's flasks. No less than 3 samples of conditioned media from different cell monolayers were examined.

RESULTS

Selection of LS-Cells and Their in Vivo Growth Properties. One of the nonmalignant sublines of the L-cell has been maintained in our laboratory for a long time. All attempts to transplant these cells to syngeneic animals have failed, even when the animals were X-irradiated (350 R) or the challenge dose reached 10 × 10⁶ cells/mouse. It is known that selection of cells for resistance to some immune reactions results in an increase in their malignancy (13, 25, 46). Immunological control over tumors is believed to be regulated by cellular immunity. Therefore, in order to obtain malignant cells from L-cells, we tried to subject them to selection for resistance to cell-mediated immune reactions. Multiple treatment of L-cells with alloimmune lymphocytes in vitro was ineffective. However, cultivation of L-cells in the peritoneal cavity of allogeneic nonimmune BALB/c mice for 3 days brought desirable results. The in vitro L-cells that survived and propagated caused tumors in a number of C3Hf mice after s.c. inoculation. Using one of these tumors, 8 passages in vivo in syngeneic mice were carried out. As a result, the level of progressive outgrowth was stabilized and amounted to 50 to 70% when 1 × 10⁶ cells/mouse were administered. However, the latent period and the tumor growth rate ranged significantly in different animals, in spite of the fact that the population was inbred. Considering this phenomenon and the possibility of cell selection in the process of cellular multiplication in the recipient organism, we decided to study cells from tumors of various sizes which developed in C3H mice after s.c. inoculation of selected LS-cells. Our hypothesis was that this would reveal changes which appear in LS-cells independently of the host. With this hypothesis in mind, 7 continuous cultures of LS-cells, designated LS1 through LS7, were established, each culture having been taken from a different tumor-bearing mouse. LS1 cells were from the lightest tumor and LS7 cells were from the heaviest tumor. LS2 culture cells did not grow in syngeneic animals as well as the initial culture of L-cells (Table 1). Thus, in subsequent experiments, a series of immunological parameters were studied both in nonmalignant L- and LS2 cells and in malignant LS1 and LS3 through LS7 cells. The malignant cells, unlike the L-cells, effectively metastasized into lungs and liver after i.v. or s.c. injections.

The inoculation of L-cells to C3H mice induced the development of cellular immunity in recipients (see below), which resulted in the protection of these animals from the subsequent challenge of malignant LS-cells (Table 2). Thus, in one of the experiments, LS1 cells were taken from 7 of 17 animals in the experimental group, and from 13 of 17 animals in the control...
group. In another experiment, LS4 cells survived in 9 of 22 animals in the experimental group and in 17 of 24 animals in the control group. These data indicate that 30% of the animals were protected. Preliminary immunization with L-cells had no effect on the transplantability of mammary tumor cells (Table 2).

Susceptibility of L- and LS-Cells to the Lytic Action of the Complement. The malignant subline of L-cells was obtained by passages of these cells in allogeneic and syngeneic animals where they could have been affected by all the types of effector cells of the immune system. Therefore, we decided to test whether the immunosensitivity of the subline to the main types of immune reactions had changed.

The sensitivity of cells to the lytic effect of the complement may vary greatly (33, 37). For this reason, the initial and selected cell cultures were compared for this characteristic. Chart 1 shows that the concentration of serum must be twice as high for the lysis of 50% of L-cells as it must be for LS1 through LS7 target cells. However, the density of surface H-2 antigens on the LS-cells is 2-fold greater than it is on L-cells (Chart 2). In fact, LS1 cells absorbed the alloantiseraum almost 2 times more easily than L-cells, even though the diameter of these cells appeared to be equal and amounted to 21.2 ± 0.75 (S.E.) and 22.2 ± 0.64 μm for L and LS1, respectively. This means that the density of H-2 antigens on LS-cells is twice as great as it is on L-cells. Therefore, it is reasonable to suppose that the sensitivity of L-cells to the complement did not change in the process of selection.

Sensitivity of L- and LS-Cells to Cytotoxicity Mediated by Alloimmune T-Lymphocytes and the Level of Lymphocyte Absorption on These Sublines. Since cellular immunity is the

Table 1
Incidence of tumors in C3Hf mice after s.c. inoculation of 1 x 10^6 L or LS1 through LS7 cells/mouse

<table>
<thead>
<tr>
<th>Tumor incidence</th>
<th>Mice were tested 20 days after inoculation.</th>
</tr>
</thead>
<tbody>
<tr>
<td>L</td>
<td>0/15</td>
</tr>
<tr>
<td>LS1</td>
<td>6/15</td>
</tr>
<tr>
<td>LS2</td>
<td>0/15</td>
</tr>
<tr>
<td>LS3</td>
<td>10/15</td>
</tr>
<tr>
<td>LS4</td>
<td>11/15</td>
</tr>
<tr>
<td>LS5</td>
<td>11/15</td>
</tr>
<tr>
<td>LS6</td>
<td>13/15</td>
</tr>
<tr>
<td>LS7</td>
<td>10/15</td>
</tr>
</tbody>
</table>

a Ratio of the number of mice with tumors to the number of mice inoculated.

Table 2
Effect of the preliminary injection of L-cells on LS1, LS4, and MMT1 tumor growth in C3Hf mice

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Challenge cells</th>
<th>No. of mice with tumors/no. of challenged cells</th>
<th>Av. tumor wt (g)</th>
<th>Experiments</th>
<th>Challenge cells</th>
<th>No. of mice with tumors/no. of challenged cells</th>
<th>Av. tumor wt (g)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>LS1</td>
<td>13/17</td>
<td>2.06 ± 0.65</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>LS4</td>
<td>17/24</td>
<td>1.37 ± 0.16</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>MMT1</td>
<td>23/25</td>
<td>0.61 ± 0.16</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>MMT1</td>
<td>26/27</td>
<td>0.79 ± 0.20</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a All control animals were inoculated with Hank’s solution on which the suspension of L-cells was prepared.
b Mice were killed on Day 30 after challenge, and the average tumor weight, including the tumors which failed to grow, was established.
c Statistics were evaluated by Student’s t test, and p values compare tumor weights.
d Mice were immunized s.c. on the back with 1 x 10^6 L-cells and 30 days later were challenged s.c. with 1 x 10^6 LS1 cells.
e Mean ± S.E.
f Mice were immunized s.c. on each foot with 1 x 10^6 L-cells and 30 days later were challenged with 1 x 10^6 LS4 cells.
g Mice were immunized with 2 x 10^6 L-cells on the back s.c. and 30 days later were challenged with 5 x 10^6 MMT1 cells.
h Mice were immunized s.c. on the back with 2 x 10^6 L-cells and 30 days later were challenged with 2.5 x 10^6 MMT1 cells.
most important determinant of transplantation and antitumor immunity (5), great attention was paid to determination of the sensitivity of both sublines to lysis mediated by various classes of effector cells. In a previous publication, we demonstrated that tumor cells may have increased resistance to allogeneic immune lymph node cells (27), i.e., to lysis by T-lymphocytes (5). Chart 3 shows that L- and LS-cells did not differ significantly in the lysis with this type of effector cells, the only exception being for LS2 cells, which were lysed significantly more strongly \( (p < 0.05 \text{ according to Student's } t \text{ test}) \). Considering that the density of H-2 antigens on the cells of the transplanted malignant subline is twice as great as it is on L-cells, it may be supposed that the sensitivity of LS1 and LS3 through LS7 cells to T-lymphocyte-mediated cytotoxicity was somewhat lower than that of nonmalignant L- and LS2-cells.

One essential property which can characterize the immunoresistance of target cells is the ability of the cells to form clusters with lymphocytes (29). However, the ability of L- and LS-cells to absorb syngeneic lymphocytes on their surfaces did not differ, and the percentage of target cells with absorbed lymphocytes varies from 12 to 14%. These data indicate that the level of cluster formation was not altered when the L-cells became malignant.

**Sensitivity of L- and LS-Cells to Cytotoxicity Mediated by Peritoneal Macrophages and Natural Killer Cells.** It is believed that macrophages (11, 20, 45) and natural killer cells (17, 22, 47) may be prominent in antitumor immunity. Chart 4 shows the sensitivity of L- and LS-cells to lysis by syngeneic peritoneal macrophages. It may be seen that cells of the malignant LS1 and LS3 through LS7 cultures and those of the nonmalignant L and LS2 cultures are lysed with equal intensity. However, quite different results were obtained when the sensitivity of different sublines of L-cells to cytolysis mediated by natural killer cells from the spleen of Wistar rats and nude mice was determined. It seems that malignant cells are significantly less susceptible to lysis by natural killer cells than nonmalignant ones \( [p < 0.01 \text{ according to Student's } t \text{ test (Charts 5 and 6)}] \).

**Effect of Conditioned Media from L- and LS-Cell Cultures on the Cytotoxic Potential of Immune Lymphocytes and Peritoneal Macrophages.** It is known that the immune resistance of tumor cells may be increased by some metabolites released by these cells into the environment. These metabolites are able to suppress the effect of immune lymphocytes and macrophages (28, 36). It may be supposed that LS-cells which become malignant have the ability to release such blocking factors. To prove this hypothesis, a comparison of conditioned media obtained from L- and LS-cell cultures was carried out. It was found that the conditioned media from cells of malignant and nonmalignant sublines either enhanced somehow the lytic action of lymphocytes and macrophages or did not influence their function (Chart 7). For comparison, the effect of a conditioned medium from a mouse mammary tumor with a high blocking activity is shown. Therefore, it can be supposed that LS cells do not differ from L-cells in this regard.

**Humoral Immune Response in C3H Mice to L- and LS-Cells and Characteristic of Anti-L-Serum.** Since the in vivo growth...
The decrease of immunogenicity to syngeneic recipients, it was necessary to compare the levels of humoral and cellular immune responses of C3H mice to L- and LS-cells. These data were necessary in order to evaluate the degree of immunological affinity between L- and LS-cells. For these reasons, the humoral and cellular immune responses in syngeneic recipients to L- and to one tumorigenic LS-cell subline was studied.

After a single immunization of mice with L-cells, no cytotoxic antibodies were found in the serum of these animals (Chart 8), nor were they found in animals with transplanted LS-cells, irrespective of whether the mice developed tumors (Chart 8). The same situation was also observed after 2-fold inoculation of L-cells. Only a 4-fold injection of L-cells resulted in the appearance of recognizable quantities of cytotoxic antibodies in the mouse serum. This serum in 1:50 dilution in the presence of rabbit complement lysed 86.0 ± 1.6% of L-cells, 87.5 ± 2.6% of LS-cells, 36.5 ± 9.9% of MMT1, mammary tumor cells, and only 15.5 ± 9.5% of cells of a primary culture of C3H embryo fibroblasts. The serum activity was not affected by absorption with rat spleen cells, but it was slightly affected by absorption with normal BALB/c spleen cells and C3H embryo fibroblasts, i.e., with cells which do not bear murine leukemia virus-associated antigens. Lymphocytes from animals infected with Gross and Rauscher viruses, as well as MMT1 cells, absorbed the serum somewhat better. However, they did not absorb the serum entirely (Chart 8). The LS-cells, regardless of the growth conditions, did not completely exhaust this serum either, while absorption with L-cells resulted in a lowering of the serum activity down to the control level. Thus, from these data, the following conclusion about the polyspecificity of the serum can be drawn: only a part of the serum activity was absorbed by cells infected with leukemia viruses. These results are in agreement with those of Leclerc et al. (32), who have also shown that the anti-L-serum is polyspecific and that LS-cells have at least 3 antigenic specificities, only 2 of which are determined by the murine leukemia viruses.

**Cellular Immune Response to L- and LS-Cells in Syngeneic Recipients.** A comparison of cellular immune response to L- and LS-cells in syngeneic animals is presented in Chart 9. Data in this chart indicate that the cytotoxic activity of effector cells from both lymph nodes and spleens did not differ after inoculation of L- and LS-cells. Assays of cytotoxicity in different effector and target cell combinations revealed that lymphoid cells, immune to L-cells, lysed the LS-cells much less effectively even though the activity was statistically significant \((p < 0.05\) according to Student’s t test). In contrast, the cytotoxicity of lymphocytes immunized against LS-cells was much weaker with respect to L-cells than with respect to LS target cells (Chart 10). These data, together with data presented in Chart 8, demonstrate that selection of L-cells for malignancy resulted in a loss of some antigens by these cells and in the acquisition of some new ones.

**DISCUSSION**

It is known that animal and human tumor cells have a wide range of malignancy. Most commonly, the malignancy of cells...
immunogenicity and immunosensitivity will significantly affect their tumor-forming ability. It has been shown that alteration of certain immunological characteristics, such as the increase of cell resistance to cellular immune reactions (42, 44), the masking of surface antigens by sialic acids (7), the shedding of surface antigens (1), or the change of cell adhesion to lymphocytes (13, 39), accompany the change in tumorigenicity and the ability to effect metastasis. Our hypothesis is also supported by the fact that athymic nude mice exhibit increased activity of natural killer cells, which confers immunity to a number of tumors in these animals (17, 43, 47).

We demonstrated in previous reports that malignant and nonmalignant cells may differ considerably in a number of immunological characteristics. Cells of the same genotype but of different tissue origin have different rates of immunogenicity in the same recipients, irrespective of the number of transplantation antigens (30). We also found that the degree of resistance of target cells to the lytic action of lymphocytes and their ability to absorb the latter range widely, again regardless of the density of transplantation antigens (29). It has been demonstrated that cells of different tumor origins are able to release substances which modify their resistance to lysis by lymphocytes and to macrophage migration (28, 38). Other authors showed that the sensitivity of cells to humoral (37) and cellular (4, 15) immune reactions does not necessarily correlate with the number of foreign antigens on their surfaces and that the resistance of different cells to the lytic action of complement (10), T-lymphocytes (2, 24), activated macrophages (20), and natural killer cells (16) may vary greatly. These data provide evidence that considerable potential possibilities of the target cells provide for a wide range of their variability in immunogenicity and immunosensitivity.

The evaluation of the above-mentioned parameters of nonmalignant and malignant sublines of L-cells has led us to conclude that cells selected for malignancy differ from parental ones only in their resistance to lysis by natural killer cells. It is noteworthy that one of the tumor cultures (LS2) displayed the characteristics of the initial line not only in terms of its transplantability but also in its sensitivity to natural killer cells. It is reasonable to suppose that transformed and potentially tumorigenic L-cells are not able to grow in syngeneic recipients, because they fail to overcome the first barrier of immunological control, i.e., the aggressiveness of natural killer cells. The validity of such a conclusion is supported by the fact that the degree of immune response in syngeneic mice to LS-cells does not exceed the degree of immune response in syngeneic mice to the L-cells.

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