Cell Adhesiveness Is Related to Tumorigenicity in Malignant Lymphoid Cells

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ABSTRACT Mouse lymphoma cells (S49) that grow in suspension culture were selected for increased tumorigenicity through continuous passages in syngeneic BALB/c mice. Developing tumors were classified as high grade malignant lymphoma, small noncleaved type. Variants were selected from these tumorigenic cells that were able to grow as a monolayer attached to their substrate, resembling, in this respect, fibroblastoid cells. Whereas the tumorigenic suspension-growing parental cells were able to induce progressive tumors with an inoculum as low as 100 cells per mouse, the adherent cells were unable to develop as tumors even at an inoculum of 1 × 10⁶ cells per mouse. In addition, mice inoculated once with live adherent cells were immunized against 1 × 10⁷ suspension-growing cells. Involvement of an immune response in the rejection of tumorigenic S49 cells was suggested by (a) adoptive transfer experiments in which spleen cells from immunized mice protected naive mice and (b) the appearance of antibodies in the sera of immunized syngeneic mice that specifically recognized both adherent and suspension-growing S49 cells and detected differences in [³⁵S]methionine-labeled antigens from these cells. Antibodies raised in rabbits against adherent cells recognized three proteins of 34,000, 61,000, and 72,000 apparent molecular weight in radiolabeled adherent cell extracts that are either absent or present in small amounts in extracts of suspension-growing tumorigenic S49 cells. These findings, taken together with our previous report (Hochman, J., A. Katz, E. Levy, and S. Eshel, 1981, Nature (Lond.), 290:248–249), suggest the S49 system as a novel system for studying growth control in malignant lymphoid cells.

The relationship among cell adhesiveness (the ability to adhere to other cells and to extracellular substrata), transformation, tumor development, and the metastatic behavior of malignant cells is a well-known but complex phenomenon that has been studied extensively (1, 3, 5, 9, 18, 20, 24) since Ludford (17) and Cowdry (6) first suggested that membranes of tumor cells had lower general adhesive properties than the normal cells from which they were derived. These previous studies have all been carried out on cells grown as monolayers (i.e., fibroblastoid) attached to their substratum and to each other.

We have undertaken to study this relationship in malignant lymphoid cells that grow attached neither to each other nor to their substratum. Our working hypothesis is that if in fibroblasts decreased cell adhesiveness correlates with increased tumorigenicity (3, 18) and vice versa, then isolation of adherent variants from malignant lymphoid cells may result in decreased tumorigenicity. Such a model would be of potential significance in the study of in vitro growth regulation of malignant lymphoid cells.

To test this approach, we have used S49 cells—a mouse lymphoma cell line of BALB/c origin (13). We have first demonstrated that stable variants can be isolated, characterized by their ability to adhere to their substratum (bottom of culture flask) and divide while attached to it (11). Using the same approach, we have subsequently isolated substrate-adhering variants from tumorigenic S49 cells, previously selected through continuous in vivo passages in BALB/c mice (12). We have also found that the adherent S49 variants demonstrated impaired tumorigenicity, while concomitantly being
able to protect the syngeneic host from subsequent challenges with tumorigenic suspension-growing S49 cells.

In the present report, we extend our previous findings by characterizing the parental S49 tumors and demonstrating that the adherent cells are directly derived from suspension-growing S49 cells. We also show that selection for adherent variants decreases their tumorigenicity by several orders of magnitude. This decreased tumorigenicity appears to be based on an immune response in the host: immune protection following a single inoculation of viable adherent cells is effective for a long period of time, the serum of the immunized mice contains antibodies that recognize both suspension-growing and adherent S49 cells, and there are antigenic differences between adherent and suspension-growing cells based on immunoprecipitation of $[^{35}]$methionine-labeled cell extracts with syngeneic mouse antisera. For the immunoprecipitations with antiserum from syngeneic mice which had been inoculated with $2 \times 10^7$ live adherent S49 cells, $1 \times 10^7$ cpm/ml for adherent cells. Antisera to S49 parental cells and $1.3 \times 10^7$ cpm/ml for adherent cells. Antisera to S49 parental cells and adherent cells were prepared by repeated subcutaneous inoculation of formalin-fixed cells in Freund's incomplete adjuvant in rabbits. Immunoprecipitations were used. Goat anti-murine leukemia virus was from the Bureau of Biologics. Immunoprecipitated extracts were exposed to SDS PAGE according to the method of Laemmli (16) with a 5% stacking gel and a 10% separating gel. Fluorography of the radiolabeled samples was done according to Bonner and Laskey (2). A series of $^{14}$C-labeled protein standards (Amersham Corp., Arlington Heights, IL) provided molecular weight markers.

RESULTS

Derivation of Tumorigenic S49 Cell Lines

In our study, S49 cells propagated in suspension culture were readapted to grow as transplanted tumors in BALB/c mice, from which they were originally derived. Selection for cells with increased tumorigenicity was carried out through serial passages in BALB/c males (Fig. 1). Thus, at the first passage only one mouse out of five developed a tumor within 32 d postinoculation, whereas at the 25th passage, tumors appeared within 7–10 d and all mice died within 13–15 d postinoculation. For further experimentation, we have used sublines derived from tumors of the 25th and subsequent passages, respectively. Whereas the above-mentioned inoculations were performed intraperitoneally, we have found that both intravenous and subcutaneous inoculation resulted in tumor development as well (data not shown).

Tumor Histopathology

Extensive involvement by grayish tumorous tissue was found in many organs of the dead mice. The tumorous masses infiltrated the mesentery, the peritoneum, and the peritoneal cavity causing ascites. The tumor invaded the muscles of the extremities and abdomen producing large masses beneath the skin. The most involved organs were the pancreas, small and large intestine, epididymis, testes, and thymus. The spleen, meninges, and brain were spared.

The histological picture of the tumor was that of a diffuse high grade malignant lymphoma with a prominent "starry sky" appearance (Fig. 2). The lymphoma cells had little cytoplasm with oval nuclei containing a few prominent nucleoli. Many cells revealed small cytoplasmic vacuoles that stained positively for fat. Many lymph nodes, as well as the spleen, were often seen surrounded by the malignant lymphoma but without infiltration into the organs themselves. The histological picture according to Rappaport's classification (21) is that of malignant lymphoma, undifferentiated. According to the new formulation (19), it is a high grade malignant lymphoma, small noncleaved type.

MATERIALS AND METHODS

Cells: Parental S49 cells (maintained in stationary suspension culture) and the substrate-adhering variants derived from them were grown in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated horse serum, penicillin (50 U/ml), and streptomycin (50 µg/ml). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂. Viability was measured using trypan blue exclusion.

Tumors: Unless otherwise specified, 1–2 x 10⁶ cells were inoculated intraperitoneally into 8–10-wk-old male BALB/c mice from which the S49 cells were originally derived (13). Mice were checked daily. The first day that tumors could be recognized was recorded. Mice were obtained from the breeding colony of the Hebrew University. They were kept in groups of six to ten per cage and fed Purina Chow and water ad lib.

Adherent Cells: Selection of substrate-adhering variants from suspension-growing tumorigenic parental cells was carried out as previously described (11) through continuous selection and enrichment of spontaneously appearing adherent cells.

Adoptive Transfer: Spleen cells from BALB/c mice that were previously (6–90 d) inoculated with a single dose of adherent S49 cells were inoculated intraperitoneally (∼10⁶ cells) into BALB/c mice 30 min before an intraperitoneal inoculation of 1 x 10⁶ suspension-growing tumorigenic S49 cells. Spleen cells from naive mice were used as controls. Mice were checked as described above.

Fluorescence-activated Cell Sorter Analysis: Cell-sorter analysis was carried out on sera derived from BALB/c mice immunized with 1 x 10⁷ live adherent S49 cells as described in the legend to Table I and unimmunized BALB/c controls. All sera were incubated with suspension-growing as well as adherent cell populations (45 min at 4°C) extensively washed with Hank's solution (without phenol red) and incubated with fluorescein-conjugated goat anti-mouse IgG (Cappel Laboratories, Inc., Conchranville, PA) for 45 min at 4°C. Samples were analyzed with the FACS-II cell sorter (Becton Dickinson Immunocytometry Systems, Oxnard, CA). Fluorescence units (F.U.) represent the median (corrected for a gain of 16) of the curve generated when cell numbers are plotted versus fluorescence intensity.

Immunoprecipitations: [$^{35}$]Methionine-labeled S49 parental and adherent cells were prepared as described by Steinberg and Coffino (23). Detergent-soluble extracts for immunoprecipitation were prepared in buffer A (0.15 M NaCl, 0.5 M Tris-HCl, pH 7.4, 0.5% Nonidet P-40, 0.05% SDS, 2 mM EDTA, and 1% aprotinin [Sigma Chemical Co., St. Louis, MO]) and had a specific activity of 4.2 x 10⁶ trichloroacetic acid-precipitable cpm/ml for S49 parental cells and 1.3 x 10⁷ cpm/ml for adherent cells. Antisera to S49 parental cells and adherent cells were prepared by repeated subcutaneous inoculation of formalin-fixed cells in Freund's incomplete adjuvant in rabbits. Immunoprecipitations were described by Gottstein and Cabral (8) using the Staphylococcus aureus method of Ketler (14). Aliquots of each extract containing 5 x 10⁶ cpm were immunoprecipitated for the immunoprecipitations with rabbit antisera. For the immunoprecipitations with antisera from syngeneic mice which had been inoculated with 2 x 10⁶ live adherent S49 cells, 1 x 10⁷ cpm...
FIGURE 2  Histological picture of S49 tumor cells. (A) High-grade malignant lymphoma, small noncleaved type showing prominent “starry sky” appearance. (B) Same tumor mass. The lymphoma cells show little cytoplasm with a few prominent nucleoli. Many macrophages show small tingible bodies (arrows). (C) Skin infiltrated by tumor cells showing destruction of the skin adnexa. (D) Small intestine infiltrated by masses of tumor cells. (E) Striated muscle extensively infiltrated by the tumor mass. H and E. × 400. All except B, hematoxylin and eosin. (A, C, and D) × 105; (B) × 660; (E) × 400.
Selection and Characterization of Adhesive Variants

The suspension-growing subline established from the 25th in vitro passage of S49 tumor cells has been designated T-25. To study the role of cell adhesiveness in tumorigenicity of lymphoid cells, we have selected for and isolated a nonmutagenized, spontaneous subline (designated T-25-Adh) characterized by its ability to adhere to the bottom of tissue culture flasks in the presence of serum (12). Upon adhesion to the substrate, these cells continue to divide until a dense monolayer is formed. At this stage daughter cells start to appear and proliferate in suspension as single cells or small aggregates of a few cells in the medium. When the suspension-growing cells derived in this manner are transferred to another culture flask, they adhere to the substrate with the same kinetics as their parental adherent cells (data not shown). These observations suggest that the predominant interactions of T-25-Adh cells are cell to substrate and not cell to cell. The substrate adhesiveness of T-25-Adh cells (and all other adherent sublines derived so far) is a stable trait insofar as continuous in vitro growth for over 18 mo does not affect their substrate adhesiveness or their adherent characteristic. It is noteworthy that the generation time of T-25-Adh cells is similar to the generation time of T-25 cells (18–20 h).

To demonstrate that T-25-Adh cells are derived from suspension-growing S49 cells and are not derived from host cells, we took advantage of the fact that S49 cells contain a prominent cytoplasmic marker system that resembles tubuloreticular structures, which can easily be distinguished at the electron microscopic level (10) and is not found in normal BALB/c tissues. These tubular structures are characteristic of suspension-growing S49 cells as well as tumors and suspension-growing sublines derived from them. We have found by electron microscopic analysis that 15/15 clones of S49 adherent variants demonstrate the same system.

When T-25 and T-25-Adh cells were compared in their ability to develop tumors, a striking difference was found (Fig. 3). Whereas in suspension-growing T-25 cells the lowest inoculum that still gave progressive tumors was $10^2$–$10^3$ cells per mouse (at that inoculum, tumors developed within 30–45 d and mice died within 45–55 d, respectively), with T-25-Adh cells, tumors did not develop even at an inoculum of $10^8$ cells per mouse. These mice were followed for up to 270 d postinoculation to exclude tumor dormancy. These findings demonstrate that the selection for substrate-adhering S49 cells significantly impairs their tumorigenic potential in BALB/c mice.

Immunogenicity of Adherent Cells

When BALB/c mice that had previously received a single injection of $10^7$ T-25-Adh cells were challenged with a highly tumorigenic dose ($10^7$ cells) of T-25 cells, it was found (Fig. 4) that no tumors developed in these mice for at least 6 mo. These findings demonstrate that T-25-Adh cells can confer upon a syngeneic host immunity against tumorigenic suspension-growing T-25 cells. These experiments were repeated using different, independently selected adherent variants, derived from clones 24.3.2 and 24.6.1 (4), with the same results. In contrast, both T-25 and T-25-Adh cells develop progressive tumors in athymic (nu/nu) mice.

Quantitative Differences in Antigens between Adherent and Suspension-growing Cells

To define molecular parameters that might be involved in the biological differences between T-25 and T-25-Adh cells, we raised antibodies against both formalin-fixed cell types in rabbits. [35S]methionine-labeled extracts from both T-25 and T-25-Adh cells were subjected to immunoprecipitation with the homologous and heterologous antisera followed by gel electrophoresis and autoradiography (see Materials and Methods). The findings shown in Fig. 5 can be summarized as
follows: One of the antisera (against T-25-Adh cells) recognizes three proteins of approximate molecular weight 34,000, 61,000, and 72,000 (lane 10, arrows) from T-25-Adh cells. This antiserum does not recognize these proteins in T-25 extracts (lane 8) nor does antiserum to T-25 cells recognize these proteins in T-25-Adh cells (lane 6). They are not major cell proteins (e.g., lanes 1 and 2). These results suggest that the T-25-Adh cells have additional antigens not present (or present only in small amounts) in the T-25 parental extracts. In parallel experiments (data not shown), T-25-Adh extracts were immunoprecipitated with anti-MuLV antisera which recognize gp70 and other antigens in these extracts. In this case, the gp70 observed migrated faster on SDS PAGE gels than the 72,000-mol-wt antigen detected with our anti-T-25-Adh rabbit serum.

To determine whether T-25 (suspension-growing) cells contain any antigens that cross-react with the 34,000-, 61,000-, and 72,000-mol-wt antigens detected on T-25-Adh cells, we had the rabbit antiserum, which recognizes these antigens, adsorbed with an excess of T-25 cells. Fig. 6 shows the results

| Table 1 |
| --- |
| **Cell-sorter Analysis of Interaction between Various Cell Types and Sera of Both Control and Immunized BALB/c Mice** |

| Cells | Normal mouse serum | T-25-Adh cells* | T-25-Adh + T-25 cells† |
| --- | --- | --- | --- |
| T-25 | 58 ± 5 (10) | 495 ± 265 (7) | 1,042 ± 750 (11) |
| T-25-Adh | 66 ± 29 (9) | 1,426 ± 510 (7) | 2,766 ± 1,570 (10) |
| BALB/c spleen cells | 19 ± 14 (6) | 13 ± 3 (5) | 20 ± 13 (7) |
| BALB/c fibroblasts | 64 (1) | — | 64 (1) |
| B10, BALB/cH2 dm2, B10D2, B10BR | 48 (1) | — | 48 (1) |

Fluorescence units (F.U.) are defined in Materials and Methods. The numbers represent the mean ± SD of each set of experiments. The number in parenthesis is the number of independent experiments (different mice). B10, BALB/cH2 dm2, B10D2, and B10BR are spleen cells from various mouse strains.

* Mice were immunized with 10⁷ T-25-Adh cells and bled after 3 wk.
† Immunized mice were primed with 10⁷ T-25-Adh cells, challenged 1 mo later with 10⁷ T-25 cells, and bled after 3 wk.

**Figure 5** Immunoprecipitation of three proteins specific to Adh* cells by rabbit antisera. Immunoprecipitations were carried out as described under Materials and Methods using antibodies to S49 parental and Adh* cells elicited in rabbits. (lane 1) 1 × 10⁶ cpm of S49 parental lysate, no immunoprecipitation; (lane 2) 1 × 10⁶ cpm of Adh* lysate, no immunoprecipitation; (lane 3) preimmune serum to S49 cells (No. 2556), S49 lysate; (lane 4) antiserum to S49 cells (No. 2556), S49 lysate; (lane 5) preimmune serum to S49 cells (No. 2556), Adh* lysate; (lane 6) antiserum to S49 cells (No. 2556), Adh* lysate; (lane 7) preimmune serum to Adh* (No. 2558), S49 lysate; (lane 8) antiserum to Adh* cells (No. 2558), S49 lysate; (lane 9) preimmune serum to Adh* cells (No. 2558) Adh* lysate; and (lane 10) antiserum to Adh* cells (No. 2558). Adh* lysate. Lanes 1 and 2 show lighter photographic exposures to allow visualization of the major proteins in whole-cell extracts. The labels at the right indicate the position of the three protein bands (molecular weight × 10⁻³) specific to the anti-Adh* serum and Adh* extracts.
of such an experiment in which the T-25 cells completely adsorbed the immunoprecipitating activity present in the anti-T-25-Adh antiserum. This result argues that these Adh antigens are only quantitatively elevated on the T-25-Adh cells and suggests that these antigens have a surface localization, since intact T-25 cells were used for the adsorption.

Sera from BALB/c mice immunized with $2 \times 10^7$ live T-25-Adh cells were also found to detect differences in proteins immunoprecipitated from T-25 and T-25-Adh extracts. As shown in Fig. 7, lanes 3 (T-25-Adh) and 4 (T-25), the 72,000- and 34,000-mol-wt antigens observed with the rabbit antiserum were also detected with the mouse antiserum, but the 61,000-mol-wt antigen was not. Several other differences between T-25 and T-25-Adh cells were seen as well, and the significance of these changes is currently under investigation.

**DISCUSSION**

Our results demonstrate that in S49 mouse lymphoma cells, an inverse correlation (similar to the one in fibroblastoid cells) exists between cell adhesiveness and tumorigenicity in syngeneic BALB/c mice. Taken together with the immunizing ability of the adherent variants, these findings suggest that our system is a unique model to study the molecular parameters involved in the relationship between cell adhesiveness and growth regulation in malignant lymphoid cells.

Although the general applicability of our approach is not yet known (and other malignant lymphoid cells are under study), Fogel and Schirrmacher (7) have recently isolated substrate-adhering variants from a metastatic lymphoma. They found that the adherent variants lost their metastatic potential. Since the selection for adherent variants is in most

**FIGURE 6** Adsorption of anti-T-25-Adh serum with T-25 cells. Rabbit antiserum to T-25-Adh cells (No. 2558), 0.5 ml, was adsorbed three times for 1 h at 4°C with $10^7$ T-25 cells which had been washed in PBS. $[^{35}S]$methionine-labeled extracts of T-25 and T-25-Adh cells were prepared and immunoprecipitated with adsorbed or unadsorbed sera as described in Materials and Methods. Adsorbed and nonadsorbed antisera were lyophilized and reconstituted before use: (lane a) 5 $\mu$l of unlyophilized antiserum, T-25 extract; (lane b) 5 $\mu$l of lyophilized preimmune serum, T-25-Adh extract; (lane c) 5 $\mu$l of lyophilized unadsorbed antiserum, T-25-Adh extract; (lane d) 5 $\mu$l of lyophilized adsorbed serum, T-25-Adh extract; and (lane e) 5 $\mu$l of lyophilized adsorbed serum, T-25 extract.

**FIGURE 7** Immunoprecipitation of proteins from T-25 and T-25-Adh cells by sera from BALB/c mice. Mice were immunized with $2 \times 10^7$ live T-25-Adh cells and immunoprecipitations were carried out as described in Materials and Methods using $10^7$ trichloroacetic acid-precipitable cpm of $[^{35}S]$methionine-labeled extracts. (lane 1) T-25-Adh extract with normal (BALB/c) mouse serum; (lane 2) T-25 cell extract with normal (BALB/c) mouse serum; (lane 3) T-25-Adh cell extract with immune BALB/c mouse serum; (lane 4) T-25 cell extract with immune BALB/c mouse serum; (lane 5) T-25-Adh cell extract with goat anti-murine leukemia virus serum.
probability a multistep event, it is conceivable that the variants they isolated have not undergone the complete chain of events leading to impaired tumorigenicity. If that is the case, it would argue against a direct genetic linkage between cell adhesiveness and decreased tumorigenicity. Further studies (complementation as well as revertant analysis) are required to clarify this question. However, irrespective of the genetic linkage between the above-mentioned traits, our findings offer a possible new approach for the xenogenization (15) of malignant lymphoid cells.

Our preliminary adoptive spleen cell transfer experiment suggests that protection is mediated by an immunologic process that has a cellular basis. More extensive experimentation will be required to determine what functional cell type mediates protection. On the basis of flow microfluorometry, there appears to be some antibody activity specific for both T-25 and T-25-Adh cells (Table I). In contrast, these antibodies do not react with spleen cells from BALB/c or other mice strains. Furthermore, greater antibody activity may have been seen on the T-25-Adh than on the T-25 target cells. This is in accordance with the immunoprecipitations carried out with the rabbit antisera against T-25-Adh cells (Figs 5 and 6) and with immunoprecipitations using mouse antiserum (Fig. 7). These findings suggest that the difference in the biological characteristics between adherent and suspension-growing S49 cells is related to the enhanced expression or a different distribution of a set of proteins in the adherent cells, thereby affecting both their tumorigenic potential and their immunizing ability.

The decreased expression of the 34,000-, 61,000-, and 72,000-mol-wt proteins in T-25 cells is interesting in view of recent findings by Reading et al. (22) that malignancies of metastatic murine lymphosarcoma cell lines correlate with decreased cell surface display of RNA tumor virus envelope glycoprotein gp70. In this study we have found that the 72,000-mol-wt protein is probably not related to gp70 expressed by murine leukemia virus in S49 cells, but these results do not rule out a relationship of the 72,000-mol-wt antigen to gp70 from another endogenous RNA tumor virus in S49 cells. Recent results from our laboratory indicate that T-25-Adh cells have an altered form of cytoplasmic tubular structures after inoculation into syngeneic mice which closely resembles viral particles, suggesting that increased expression of viral antigens could play a role in the decreased tumorigenicity of T-25-Adh cells (J. Hochman and N. Mador, unpublished observations).

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