Shedding and Immunoregulatory Activity of YAC-1 Lymphoma Cell Gangliosides

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ABSTRACT

YAC-1 lymphoma cells, both when cultured in vitro and when passaged in ascites form in vivo, synthesize gangliosides (means of 22.1 and 14.7 nmol lipid-bound sialic acid isolated per 10⁶ cells, respectively) with potent inhibitory effects on mitogen- and antigen-induced lymphoproliferation: 10 to 30 nmol highly purified YAC-1 gangliosides/ml caused >90% inhibition of proliferative responses of murine lymphocytes to concanavalin A, lysozyme (a soluble specific antigen), and allogeneic cells (mixed-lymphocyte response). Measurable quantities of these gangliosides were shed by the tumor cells in vitro and also were recovered from the ascites fluid in vivo. Furthermore, the gangliosides isolated from ascites fluid (mean of 15.3 nmol/ml) had inhibitory activity of a magnitude similar to that of the gangliosides isolated from the tumor cells. Therefore, significant inhibition of normal lymphoproliferative responses by tumor-derived gangliosides occurred at ganglioside concentrations which are actually present in the fluid surrounding the tumor cells in vivo. These results support the hypothesis that shedding of gangliosides may serve to protect tumor cells from host immune destruction.

INTRODUCTION

 Certain gangliosides, which are sialic acid-containing glycosphingolipids, are known to have potent immunoregulatory properties, including inhibitory effects on antibody production in vivo (1), on the generation of antibody-synthesizing cells in vitro (22), and on lymphocyte proliferative responses to mitogens and antigens in vitro (17, 33). Both bovine and human brain gangliosides, and gangliosides shed by antigen-stimulated lymphocytes (4) have been shown to exhibit such immunoregulatory properties.

 The hypothesis that shedding of gangliosides may contribute to the escape of tumor cells from immune destruction in vivo (5) is supported by the above findings and by observations of elevated levels of circulating gangliosides in both animals and humans with cancer (8, 16, 23, 28). It has further been suggested, although not proven, that tumor-derived gangliosides may be a cause of generalized immunosuppression frequently observed in tumor-bearing hosts (17).

 To directly test the hypothesis that purified gangliosides, isolated from homogeneous tumor cell populations, may have such immunoregulatory activity, we have studied a murine tumor system, the YAC-1 lymphoma. We have found: (a) that YAC-1 cells cultured in vitro synthesize gangliosides which inhibit mitogen- and antigen-induced lymphoproliferative responses and which are actively shed by these cells; and (b) that inhibitory, chromatographically similar gangliosides are also found in YAC-1 cells propagated in vivo and in the ascites fluid surrounding these cells. Together, the results provide direct evidence supporting an immunoregulatory role of gangliosides synthesized by tumor cells in vivo.
for 10 days. Six hr later, the cells were harvested, and gangliosides were gel filtration to remove low-molecular-weight contaminants (32). Samples previously found to efficiently separate less polar lipids from gangliosides in Click's medium with 0.5% syngeneic mouse serum (19), supplemented isolated, freed of contaminating erythrocytes by hypotonic lysis, washed, and resuspended in complete medium consisting of Dulbecco's modified Eagle's medium with 4.5 g glucose/liter (Grand Island Biological Co., Grand Island, N. Y.) and 5% heat-inactivated PCS, supplemented with 25 in vitro or in vivo during the 24-month period of these studies.

To exclude the possibility that the cell ganglioside patterns reflected cell adsorption of gangliosides present in the FCS of either in vitro or in vivo during the 24-month period of these studies.

To exclude the possibility that the cell ganglioside patterns reflected cell adsorption of gangliosides present in the FCS of the cell culture medium or, in vivo, derived from the host and present in the ascites fluid, we documented the incorporation of radiolabeled precursor sugars into newly synthesized gangliosides both in vitro and in vivo. As seen in Fig. 2, the autoradiographic pattern in each experiment was identical to the ganglioside pattern visualized by resorcinol staining, confirming that the

**RESULTS**

**Characterization of YAC-1 Cell Gangliosides.** Four major gangliosides, with TLC mobility between G_{M0} and G_{D0} standard brain gangliosides, were seen on TLC of the gangliosides isolated from YAC-1 cells (Fig. 1). At least 8 other minor bands were also visible, suggesting a high degree of structural diversity of the cell gangliosides. The patterns of gangliosides isolated from cells cultured in vitro and from cells passaged in vivo were very similar (Fig. 1), indicating that the ganglioside profile of YAC-1 cells is independent of their propagation in vitro versus in vivo.

Each separate preparation of YAC-1 cell gangliosides was quantitated and qualitatively characterized by TLC, because variants of YAC-1 cells have been found to have strikingly different ganglioside patterns (36). The ganglioside content of cells cultured in vitro \((n = 7)\) or in vivo \((n = 6)\) \([22.1 ± 3.7 (S.E.) versus 14.7 ± 4.7 nmol LBSA per 10^6 cells, respectively\] was not significantly different \((p > 0.2)\). Moreover, the TLC mobilities of the major bands isolated from the individual preparations were very reproducible, suggesting that no YAC-1 variants had developed either in vitro or in vivo during the 24-month period of these studies.

Shedding and Immunoregulation by Tumor Cell Gangliosides

Gangliosides were dissolved in complete medium, sonicated under N_2 for 2 min at room temperature in a Branson bath sonicator (Scientific Products, Irvine, Calif.), and added to the cell suspensions immediately before (HEL and MLR) or 18 hr before (Con A) addition of the stimulant. Final culture volumes, in flat-bottomed 96-well Microtiter plates (Flow Laboratories, Ingelwood, Calif.), were 100 ml for the Con A and 200 ml for the HEL and MLR assays. Cell and stimulant concentrations \((\mu l)\) were 1 X 10^6/2 for D_2/2 splenocytes (Con A, 3 µg), 2 X 10^6 lymph node cells (HEL, 100 µg), and 1 X 10^6 each of D_2/2 and A/Sn splenocytes in the MLR. The plates were incubated at 37° in a humidified atmosphere of either 5% CO_2:95% air (72 hr for Con A-induced proliferation and 120 hr for the MLR) or 2% CO_2:98% air (for 120 hr for HEL-induced proliferation). Cultures were pulsed with 0.5 to 1 µCi \([\text{T}^3\text{H}]\)thymidine \((\text{specific activity}, 6.7 

**Lymphocyte Proliferation Assays.** YAC-1 lymphoma gangliosides were tested for modulatory effects on the proliferative responses of murine lymphocytes to the mitogen, Con A, to allogeneic cells \((\text{bidirectional MLR}), and to a soluble specific antigen, HEL.

Splenocytes of D_2/2 and A/Sn mice \((\text{The Jackson Laboratory})\) were isolated, freed of contaminating erythrocytes by hypotonic lysis, washed, and resuspended in complete medium consisting of Dulbecco's modified Eagle's medium with 4.5 g glucose/liter \((\text{Grand Island Biological Co., Grand Island, N. Y.})\) and 5% heat-inactivated FCS, supplemented with 25 µmol 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 0.07 µmol 2-mercaptoethanol, penicillin \((50 \text{ units/ml})\), and streptomycin \((50 \mu g/ml)\). Lymph node cells were isolated from inguinal, periaortic, and peritoneal nodes of female C57BL/6 mice at 9 days after immunization with 100 µg HEL. These cells were suspended in complete medium consisting of Click's medium with 0.5% syngeneic mouse serum (19), supplemented as above. Cell viability was consistently >90%.

Gangliosides were dissolved in complete medium, sonicated under N_2 for 2 min at room temperature in a Branson bath sonicator (Scientific Products, Irvine, Calif.), and added to the cell suspensions immediately before (HEL and MLR) or 18 hr before (Con A) addition of the stimulant. Final culture volumes, in flat-bottomed 96-well Microtiter plates (Flow Laboratories, Ingelwood, Calif.), were 100 µl for the Con A and 200 µl for the HEL and MLR assays. Cell and stimulant concentrations \((\mu l)\) were 1 X 10^6/2 for D_2/2 splenocytes (Con A, 3 µg), 2 X 10^6 lymph node cells (HEL, 100 µg), and 1 X 10^6 each of D_2/2 and A/Sn splenocytes in the MLR. The plates were incubated at 37° in a humidified atmosphere of either 5% CO_2:95% air (72 hr for Con A-induced proliferation and 120 hr for the MLR) or 2% CO_2:98% air (for 120 hr for HEL-induced proliferation). Cultures were pulsed with 0.5 to 1 µCi \([\text{T}^3\text{H}]\)thymidine \((\text{specific activity}, 6.7 

**Statistical Analysis.** Student's t test was used to determine the significance of differences between mean ganglioside concentrations.
gangliosides isolated from the cells had actually been synthesized by the cells.

**Shedding of Gangliosides by YAC-1 Cells.** In a series of experiments, gangliosides in conditioned medium, generated by the 72-hr culture of YAC-1 cells in vitro, and in control unconditioned medium (i.e., FCS gangliosides) were isolated and quantitated as described in "Materials and Methods." A net ganglioside concentration of 0.36 ± 0.14 nmol LBSA per ml medium (n = 3), due to shedding of gangliosides by tumor cells, was found. Estimating the average cell concentration during the 72 hr to have been 1 ± 10⁶ per ml, these results indicate a calculated approximate shedding rate of 12 nmol LBSA per 10⁶ cells/24 hr, or about 50% of the cell ganglioside content per 24 hr. The pattern of gangliosides isolated from the conditioned medium showed striking similarity to the pattern of the major cell-synthesized gangliosides (Fig. 3). The additional bands of higher mobility not seen in the cell ganglioside pattern (Fig. 3) were derived from the FCS which contains Gm2 as the major ganglioside component. Together, the quantitative and qualitative findings demonstrate substantial shedding of cell gangliosides by YAC-1 cells cultured in vitro.

In the ascites fluid surrounding YAC-1 cells propagated in vivo, the quantity of ganglioside present (15.3 ± 1.7 nmol/ml; n = 4) was much higher than in the in vitro system. To the extent that these gangliosides were shed by the tumor cells, this higher concentration may be a reflection of an approximately 100-fold higher cell density (i.e., 2 to 3 × 10⁶/ml) in vivo. As in the in vitro system, the TLC pattern of gangliosides isolated from the ascites fluid included the major bands seen in the TLC pattern of gangliosides isolated from the cells passaged in vivo (Fig. 3). The most prominent ganglioside in the ascites fluid, with TLC mobility slightly greater than that of Gm2, ganglioside, was, however, only faintly visible on the TLC of the cell gangliosides (Fig. 3). Possible explanations for this difference in the relative proportions of specific gangliosides found in the cells and in the surrounding in vivo fluid remain to be investigated. They would include: (a) alteration of cell shedding of gangliosides when cells are passaged in vivo instead of in vitro; (b) presence of gangliosides derived from serum or other host tissue, rather than shed by the tumor cells; and (c) biochemical metabolism of the gangliosides once shed into the ascitic fluid. Whatever the basis for the substantial levels of gangliosides in the ascites fluid, these findings imply that there is a significant ganglioside concentration in the local environment of the tumor cells in vivo.

**Modulation of Lymphocyte Proliferative Responses by Tumor Cell Gangliosides.** Modulation of the proliferative response of normal murine splenocytes to the mitogen, Con A, by gangliosides isolated from YAC-1 lymphoma cells is shown in Chart 1. A dose-related inhibition of this lymphocyte proliferative response was observed. Concentrations of 25 to 30 nmol YAC-1 cell gangliosides/ml, or 2.5 to 3 nmol/2 × 10⁵ splenocytes/culture, caused 90% inhibition of net [³H]thymidine uptake by stimulated splenocytes. This marked inhibitory activity was characteristic of gangliosides isolated both from YAC-1 cells cultured in vitro and those passaged in vivo (Chart 1). Interestingly, gangliosides isolated from the ascites fluid surrounding YAC-1 cells proliferated in vivo also markedly inhibited lymphocyte proliferation (Chart 1).

**Fig. 2. Autoradiography of YAC-1 lymphoma cell gangliosides.** TLC patterns of radio labeled YAC-1 lymphoma cell gangliosides were assessed both by resorcinol-HCl staining (Lanes A to C) and by autoradiography of the same plate (Lanes D and E). Gangliosides of YAC-1 cells cultured in vitro (4.4 nmol LBSA; 8000 cpm) visualized by resorcinol-HCl staining (Lane B) and by autoradiography (Lane D), and of YAC-1 cells propagated in vivo (5 nmol LBSA; 8000 cpm) visualized by resorcinol-HCl staining (Lane C) and by autoradiography (Lane E) are shown. Lane A, Gm2 and bovine brain standard gangliosides (5 nmol LBSA). Bands above Gm2 in Lane C are resorcinol negative; all other bands in Lanes A to C are resorcinol positive.

**Fig. 3. Shedding of YAC-1 lymphoma cell gangliosides.** Gangliosides of YAC-1 lymphoma cells cultured in vitro (Lane B) or propagated in vivo (Lane D) are compared with gangliosides isolated from the respective surrounding fluid obtained in the same experiment: conditioned medium (Lane C) and ascites fluid (Lane E). Lanes A and F, Gm2 and bovine brain standard gangliosides. Each lane contained 10 nmol LBSA. Both thin-layer chromatograms (Lanes A to C and D to F) were stained with resorcinol-HCl. Bands above Gm2 in Lane C are resorcinol negative; all other bands are resorcinol positive.

**Chart 1.** Inhibition of Con A-induced murine lymphoproliferative responses by gangliosides isolated from YAC-1 lymphoma cells and from ascites fluid. Points, mean and bars, range (1 to 3 separate experiments, each performed in triplicate) of the change in net [³H]thymidine uptake of Con A-stimulated splenocyte cultures caused by incubation in the presence of the indicated concentration of gangliosides. A. gangliosides isolated from cells cultured in vitro (■); B, gangliosides isolated from cells propagated in vivo (△) and from the surrounding ascites fluid (▼).
YAC-1 lymphoma cell gangliosides inhibit mitogen- and antigen-induced lymphoproliferative responses

Table 1

<table>
<thead>
<tr>
<th>Ganglioside</th>
<th>Sequential purification steps&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Concentration (nmol LBSA/ml)</th>
<th>Stimulant</th>
<th>&amp;[^3H]Thymidine uptake&lt;sup&gt;2&lt;/sup&gt;</th>
<th>[3H]Thymidine uptake&lt;sup&gt;2&lt;/sup&gt;</th>
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<tr>
<td></td>
<td>Control (no added ganglioside)</td>
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<td></td>
<td>Modified method of Irwin and Irwin&lt;sup&gt;c&lt;/sup&gt;</td>
<td>30</td>
<td>Con A</td>
<td>74</td>
<td>11</td>
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<tr>
<td></td>
<td>Sephadex G-50 gel filtration&lt;sup&gt;a&lt;/sup&gt;</td>
<td>30</td>
<td>Con A</td>
<td>7.9 (89)</td>
<td>2.6 (78)</td>
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<tr>
<td></td>
<td></td>
<td>20</td>
<td>Con A</td>
<td>40 (45)</td>
<td>2.1 (90)</td>
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<tr>
<td></td>
<td></td>
<td>10</td>
<td>Con A</td>
<td>70 (5)</td>
<td>2.5 (95)</td>
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<td></td>
<td>Preparative TLC&lt;sup&gt;f&lt;/sup&gt;</td>
<td>30</td>
<td>1.3 (88)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>20</td>
<td>53</td>
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<td></td>
<td></td>
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<td>Con A</td>
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* Mean net cpm × 10<sup>-3</sup> of triplicate cultures; S.E. <10%.  
<sup>b</sup> Sequential purification procedures do not abrogate inhibitory activity.  
<sup>c</sup> Removal of phospholipids, neutral lipids, and neutral glycosphingolipids.  
<sup>d</sup> Numbers in parentheses, percentage of inhibition versus control.  
<sup>a</sup> Removal of nucleotide sugars, low molecular weight peptides, and salts; removed fraction was not inhibitory (not shown).  
<sup>f</sup> Compared to modified Irwin and Irwin method alone, the additional gel filtration and preparative TLC steps resulted in a 45-fold reduction in protein, to 0.002 mol%.

Inhibition of [3H]thymidine uptake by gangliosides was documented to reflect actual inhibition of cell proliferation, in that lack of an increase in the cell count in cultures exposed to Con A and gangliosides (30 nmol/ml) was observed, in comparison to a doubling of the cell count in cultures exposed to Con A alone. In contrast, the viability of unstimulated splenocytes (assessed by trypan blue dye exclusion) was not affected by culture in the presence of these gangliosides, thereby excluding nonspecific cytotoxicity as the cause of the inhibition. In separate experiments in which the concentration of Con A was varied from 0.75 to 50 μg/ml (15 times the optimal concentration), the degree of inhibition caused by 30 nmol YAC-1 cell gangliosides/ml was constant (85 to 90%). The lack of decreasing inhibition with increasing Con A concentration provides evidence that, as observed previously in the case of mixed brain gangliosides (17, 33), the tumor cell gangliosides did not inhibit the lymphoproliferative responses to Con A merely by direct binding to, and inactivation of, the mitogen.

The effects of YAC-1 cell gangliosides on lymphoproliferative responses to 2 other stimulants, allogeneic cells (MLR) and a soluble specific antigen (HEL), were also studied to determine whether inhibitory effects of these tumor cell gangliosides were restricted to proliferative responses induced by Con A. In parallel experiments, proliferative responses to all 3 stimulants were markedly inhibited by the YAC-1 gangliosides (Table 1). Therefore, inhibition of proliferation was not dependent on the nature of the stimulant and, thus, reflects a general inhibitory effect on mitogen- and antigen-induced lymphoproliferation. Moreover, the results in Table 1 suggest an even more marked inhibitory effect of these tumor cell gangliosides on the proliferative responses to specific antigens (i.e., MLR and HEL) than to the nonspecific mitogen, Con A, an observation also made in testing these same gangliosides in human lymphoproliferation assays (14).

Recently, it has been shown that protein contamination may account for some of the biological effects attributed to gangliosides (11). To exclude the possibility that contaminating protein might be responsible for the inhibition of lymphoproliferation, we tested the effect of sequential purification procedures on protein content and on inhibitory activity (Table 1) of gangliosides isolated from YAC-1 cells cultured in vitro. Gangliosides recovered in CMW 50:50:15 and redissolved in CM 1:1 contained approximately 4 μg protein/100 nmol LBSA. The further purification procedures described in "Materials and Methods," coupled with an additional final step, preparative TLC, resulted in a 45-fold reduction in protein content (to approximately 0.1 μg/100 nmol LBSA, or 0.002 mol% protein) in the purified gangliosides. The quantitation method (autoradiographic visualization (21) of the protein which had been 125I-labeled (20) and resolved by sodium dodecyl sulfate gel electrophoresis (21)) also demonstrated that the minute quantity of protein still associated with the gangliosides after preparative TLC was qualitatively identical (apparent molecular weight of the major band M<sub>1</sub> = 88,000) to that present at the earlier stages of the purification. This purification sequence resulted in no reduction of inhibitory effects of the YAC-1 cell gangliosides on lymphoproliferative responses to Con A, MLR, or HEL (Table 1). Therefore, the results provide very strong evidence supporting the conclusion that the inhibitory activity is due to ganglioside.

DISCUSSION

The YAC-1 lymphoma was selected as the tumor system for the present studies of tumor cell gangliosides and their effect on normal lymphocyte proliferative responses, because it had been shown that YAC-1 cells propagated i.p. in vivo release, into the surrounding ascites fluid, membrane vesicles enriched in lipid content over that of the membrane itself (24). Therefore, it seemed possible to us that immunologically active polar glycosphingolipids (gangliosides) might also be shed by these cells in substantial quantities. We investigated both the gangliosides of YAC-1 cells cultured in vitro and of YAC-1 cells proliferating in vivo: (a) in order to allow comparison of the characteristics of the cell gangliosides isolated under the 2 conditions; and (b) because in vitro system would allow isolation of gangliosides...
synthesized strictly by the tumor cells rather than by normal or reactive host cells, as might be the case in vivo; while (c) the in vivo system, by yielding data concerning ganglioside concentrations in the actual local environment of the tumor as well, might provide important information regarding the potential in vivo significance of our immunological findings.

YAC-1 cells, whether cultured in vitro or propagated in vivo, contained similar quantities of gangliosides, with similar qualitative patterns. In both cases, identity between ganglioside patterns visualized by autoradiography and by resorcinol staining documented that the cell-derived gangliosides had been synthesized de novo by the YAC-1 cells. This finding is of importance, because it suggests that the immunoregulatory activity of the gangliosides we have isolated represents a true tumor cell-associated effect, rather than effects of gangliosides in the culture medium (in vitro) or derived from the host (in vivo).

Gangliosides which we had thus shown to have been synthesized by the tumor cells in vitro were tested in lymphoproliferation assays. The marked inhibitory effect of these highly purified gangliosides, which was independent of the nature of the stimulating antigen and not due to trace contaminating protein, therefore provides an unequivocal demonstration of the immunosuppressive activity of strictly tumor cell-derived gangliosides. Using highly purified gangliosides isolated from cells cultured in vitro avoided the problem, recognized by others (12), associated with ascribing immunoinhibitory activity of gangliosides isolated from tumor-containing tissue (i.e., AKR lymphomatous thymus) to gangliosides of the tumor cells as opposed to gangliosides of other cells present in the tissue. Furthermore, the demonstration of quantitatively similar dose-related inhibition of lymphoproliferation by gangliosides isolated from YAC-1 cells which had been propagated in vivo shows that tumor cells proliferating in vivo also synthesize such immunoregulatory gangliosides.

Another major objective of our studies was to assess shedding of gangliosides in the YAC-1 system. Shedding of gangliosides by tumors has been suggested by findings of increased circulating ganglioside levels in tumor-bearing hosts (8, 16, 23, 28). More recently, the shedding of a specific tumor-associated ganglioside was documented by the binding, to sera of patients with colon carcinoma, of a monoclonal antibody found to be directed to a ganglioside specific for colon carcinoma (9, 18). In the tumor system we studied, the quantities of gangliosides shed by the tumor cells in vitro were such as to be detectable by the much less-sensitive standard TLC staining techniques, suggesting that the degree of shedding of gangliosides by tumor cells may be greater than previously recognized.

The demonstration of ganglioside synthesis and shedding by YAC-1 cells in vitro provides the background for addressing the question of the potential in vivo relevance of immunoregulatory tumor cell gangliosides, i.e., do gangliosides associated with proliferating tumor cells in vivo have immunoregulatory properties, and are these gangliosides present in biologically significant quantities in vivo? If such were the case, ganglioside shedding, which has been proposed as a possible mechanism of tumor cell escape from immune surveillance (5), could indeed, as has been suggested (17), contribute to the widely reported inhibitory effects of malignant ascites fluid and plasma of tumor-bearing hosts on immune responses such as mitogen-induced lymphoproliferation. Our results showing significant inhibitory activity of the purified gangliosides isolated from the ascites fluid surrounding the YAC-1 cells proliferating in vivo, with a very similar dose-response curve to that of the cells themselves, are in accord with this hypothesis.

Gangliosides isolated from normal tissue, such as brain, also inhibit Con-A-induced murine lymphoproliferation (17), with the degree of inhibition being quantitatively similar to that of the YAC-1 cell gangliosides we studied. This suggests that, independent of their cell of origin and molecular structure, gangliosides may inhibit lymphoproliferation by a common mechanism. However, it should be emphasized that, despite such similar inhibitory activities, an important difference does exist in vivo between brain and tumor cell gangliosides, i.e., while brain gangliosides are normally not found in the peripheral circulation, tumor cell gangliosides have been documented to be present in the local environment of the tumor (present study) and in the peripheral circulation of tumor-bearing hosts (9, 18, 23). Therefore, gangliosides shed by tumor cells would be much more likely than normal brain gangliosides to come into contact with, and significantly affect, the immune system in vivo. The potential for immunosuppression by tumor-associated gangliosides in vivo is further supported by the finding that the actual ganglioside concentrations in the malignant ascites fluid associated with the YAC-1 lymphoma are high enough to be expected to have a significant inhibitory effect on murine lymphoproliferative responses.

A direct relationship between total membrane sialic acid (protein- and lipid-bound) and metastatic potential of tumor cells has recently been suggested (37), although conflicting results had been obtained when the total cell ganglioside content was measured (27, 35). The documentation of immunoregulatory activity and of the shedding of substantial quantities of gangliosides by YAC-1 cells suggests an alternate explanation for how gangliosides might relate to metastasis, i.e., it may be that quantitative or qualitative characteristics of ganglioside shedding, rather than cell or membrane ganglioside content alone, are important factors modulating, possibly by an immunological mechanism, the metastatic potential of tumor cells.

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