DECREASED GLYCOLIPID ANTIGEN EXPRESSION IN LYMPHOMA CELL VARIANTS ESCAPING FROM ANTI-GLYCOLIPID SEROTHERAPY*

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The availability of monoclonal antibodies specific for tumor-associated cell surface markers has resulted in renewed interest in cancer serotherapy. In fact, several clinical trials have been conducted (reviewed in ref. 1), but successful tumor remission has been achieved in only one case (2). A crucial problem for specific immunotherapy is the potential escape of antigen-deficient variants from immune control. We recently described a model system in which mice could be protected from challenge with L5178Y lymphoma cells by passive infusion of a monoclonal antibody specific for the glycolipid asialo GM2 (gangliotriosylceramide; GalNacβ1 $\rightarrow$ 4Galβ1 $\rightarrow$ 4Glcβ1 $\rightarrow$ ceramide), which was present on the lymphoma cell surface (3). In a few antibody-treated mice, ascites tumor cells eventually proliferated that were deficient in the chemical quantity of asialo GM2 (3). The purpose of the present study was to demonstrate that these emergent cells were in fact variants of the cells used for challenge and to characterize the display of the glycolipid antigen on these cells.

Materials and Methods

Mice. Male DBA/2 and CBA mice (8 wk old) were obtained from The Jackson Laboratory, Bar Harbor, ME. Male (BALB/c $\times$ DBA/2)F_1 (CD2$\cdot F_1$) mice (8 wk old) were obtained from Laboratory Supply, Indianapolis, IN.

Tumor Cell Lines. The derivation of a variant of the DBA/2 lymphoma cell line L5178Y, designated subclone 1A1, has been described elsewhere (4). This variant was used as target cell for serotherapy (3) because it contained a high level of asialo GM2. During the course of serotherapy studies, ascites tumor cells were removed from both untreated and antibody-treated mice and returned to tissue culture for analysis. Cells were removed from an untreated DBA/2 mouse 71 d after challenge (designated 1A1-TU cells) and from an untreated CD2$\cdot F_1$ mouse 56 d after challenge (1A1-245 cells). The following cell lines were isolated from antibody-treated DBA/2 mice on the indicated day after challenge: 1A1-616 (day 61), 1A1-G3AF7 (day 76), and 1A1-1010 (day 100).

Antibodies. BALB/c monoclonal IgM and IgG3 antibodies specific for asialo GM2 have been characterized previously (5). Mouse alloantiserum obtained through the courtesy of Dr. John G. Ray, National Institutes of Health, Bethesda, MD, was specific for the H-2D^d end of the H-2^d complex and was designated NIH serum D-13.

Serotherapy Protocol. As previously described (3), the protocol consisted of intraperitoneal

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challenge of DBA/2 mice with 10^6 1A1 or 10^5 1A1-G3AF7 cells on day 0. Mice were treated intraperitoneally on days 1, 3, 7, and 10 with 0.1 ml of ascites fluid from mice bearing hybridoma cells that produce monoclonal IgG3 anti-asialo GM2 (3 mg antibody per ml of pooled ascites fluid). Due to lethal Sendai virus infections of DBA/2 mice in our mouse colony, the identical serotherapy protocol was also conducted in CD2·F_1 mice.

Karyotype Analysis. Chromosome preparations were made as described by Miller et al. (6). This method has been used previously for the analysis of other L5178Y lymphoma variants (7).

Effector Cells. The source of natural killer (NK) cells was the spleens of adult CBA mice that had received 100 μg poly I:C (Sigma Chemical Co., St. Louis, MO) intraperitoneally in 0.1 ml RPMI 1640 1 d earlier.

Microcytotoxicity Assays. Target cells were ^{51}Cr labeled as previously described (8), assays were performed in V-bottomed microtiter plates (Linbro Scientific, Hamden, CT), and all dilutions were made with RPMI containing 10% newborn calf serum (Sterile Systems, Logan, UT). For antibody-complement assays, each well received 0.05 ml antiserum, 0.05 ml of a 1:8 dilution of either native or heated (56°C, 30 min) English smooth hair guinea pig serum as a complement source, and 0.1 ml containing 2×10^4 labeled target cells. Plates were incubated for 45 min at 37°C, centrifuged at 250 *g* for 1 min, and 0.1 ml of the cell-free supernatant was measured for ^{51}Cr content in a gamma scintillation counter.

For NK cell assays, effector cells and 2×10^4 ^{51}Cr-labeled target cells were combined in a total volume of 0.2 ml per well. The plates were centrifuged at 250 *g* for 1 min both before and after a 4-h incubation at 37°C, and then 0.1 ml of the supernatant was assayed for ^{51}Cr content.

Specific lysis was calculated relative to the spontaneous release observed in wells containing target cells alone according to the following equation: percent specific lysis = ([test cpm − spontaneous cpm]/[cpm released by three cycles of freeze-thaw]) × 100. Freeze-thawed cells released ~80% of their total radioactivity.

Flow Microfluorometry. Cells were washed twice and then incubated for 60 min at 4°C with a 1:10 dilution of mouse IgM anti-asialo GM2 ascites fluid that had been heated at 56°, 30 min or a 1:25 dilution of anti-$H2^d$ serum (NIH serum D-13). After three washes, the cells were incubated for 60 min at 4°C with a 1:50 dilution of fluorescein-labeled goat anti-mouse immunoglobulin (N. L. Cappel Laboratories, Cochranville, PA). After two washes, the cells were analyzed on a fluorescence-activated cell sorter (FACS IV; B-D FACS Systems Becton, Dickinson & Co., Sunnyvale, CA).

Results

Cytogenetic Analysis. As previously described (3), serotherapy with IgG3 anti-asialo GM2 protected the majority of mice from challenge with an asialo GM2-positive variant (1A1) of L5178Y lymphoma cells. However, in a few cases asialo GM2-deficient ascites tumor cells arose after a prolonged period (3). To determine whether these ascites tumor cells were progeny of the cells used for initial challenge rather than having been newly induced in the host, we searched for abnormal chromosomal markers common to both original and emergent cells.

The original 1A1 cells used for challenge as well as the cells derived from two antibody-treated mice had more than the diploid number of chromosomes, often about 50–60 chromosomes per cell. These included copies of normal chromosomes, as well as structurally altered or marker chromosomes. Fig. 1 demonstrates that several marker chromosomes were found to be common to all three cell lines. These results indicate that these lines were closely related and strongly suggest that the cells arising after antibody treatment were progeny of the original 1A1 cell inoculum.

Antigen Display on Lymphoma Variants. Flow microfluorometry was used to examine the display of asialo GM2 and $H\text{-}2^d$ on 1A1 and ascites tumor cells derived from untreated and anti-asialo GM2-treated mice (Fig. 2). All cell lines were cultured in vitro for at least 1 mo before analysis to eliminate both host cells and residual

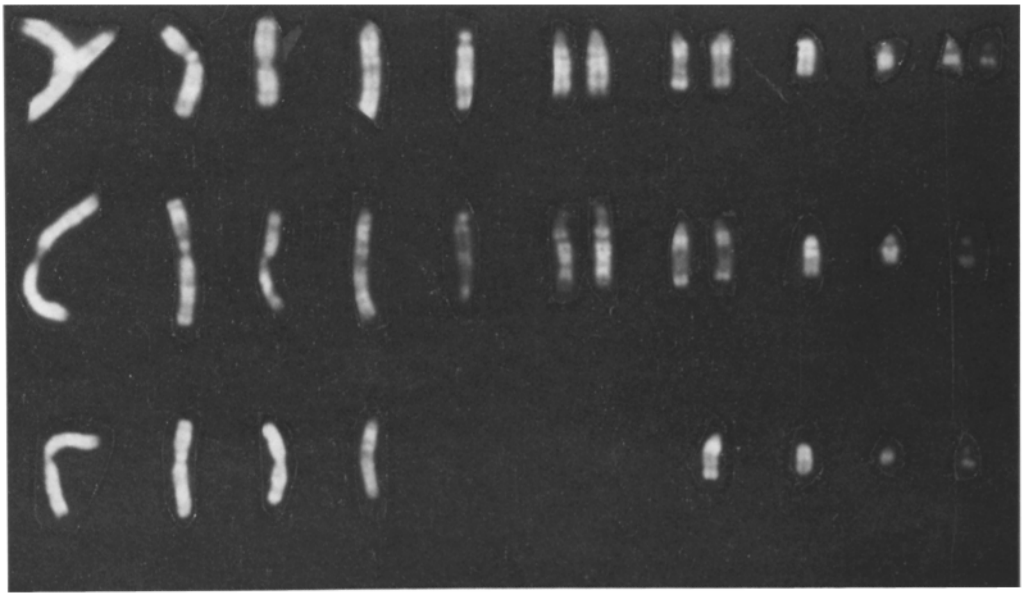

FIG. 1. Quinacrine-stained abnormal, or marker, chromosomes from L5178Y variant cell lines. Top row, subclone 1A1 cells used for challenge in serotherapy experiments. Middle row, subclone 1A1-G3AF7 cells harvested from a mouse 76 d after challenge with 10^6 subclone 1A1 cells and treatment with IgG3 anti-asialo GM2. Bottom row, subclone 1A1-616 cells harvested 61 d after challenge and treatment. The largest marker is a biarmed chromosome that includes two copies of the X (probably plus some duplicated material). The third largest is a chromosome in which both arms are copies of number 10. The sixth and seventh chromosomes from the left are identical; they are copies of number 2 with an elongated centromeric region. The eighth and ninth from the left are again identical representing a number 11 with material added to the distal end.

antibody that could have covered or modulated tumor cell surface determinants. In contrast to the uniform, positive reaction of 1A1 cells with anti-asialo GM2, <5% of cells from antibody-treated mice (1A1-G3AF7 , 1A1-616, and 1A1-1010) displayed detectable asialo GM2. Interestingly, cells from untreated mice represented a mixture of asialo GM2-positive and -negative cells; 49% of 1A1-TU cells and 25% of 1A1-245 cells reacted with anti-asialo GM2. Unlike the dramatic alterations in asialo GM2 display, the expression of H-2^d on these cell lines was virtually unchanged from that of 1A1 cells. Thus, in vivo passage of 1A1 cells appeared to result in selection of cells deficient in asialo GM2 but not H-2^d determinants, particularly in mice treated with anti-asialo GM2 (see Discussion).

To determine the stability of asialo GM2 display on the cells derived from untreated mice, 1A1-TU cells were stained with anti-asialo GM2 and then sorted on the flow cytometer to separate asialo GM2-positive and -negative populations. These cells were returned to culture for 10 d and then reanalyzed for asialo GM2 expression. Fig. 3 indicates that each population retained its original asialo GM2 display and thus represented stable subsets of cells with regard to asialo GM2 expression.

The cell variants were also compared for their susceptibility to antibody-complement-mediated lysis. The purpose of this experiment was not only to obtain additional evidence regarding the antigenic display of these cells, but also to determine their sensitivity to lytic effector mechanisms. IgM anti-asialo GM2 ascites fluid was used

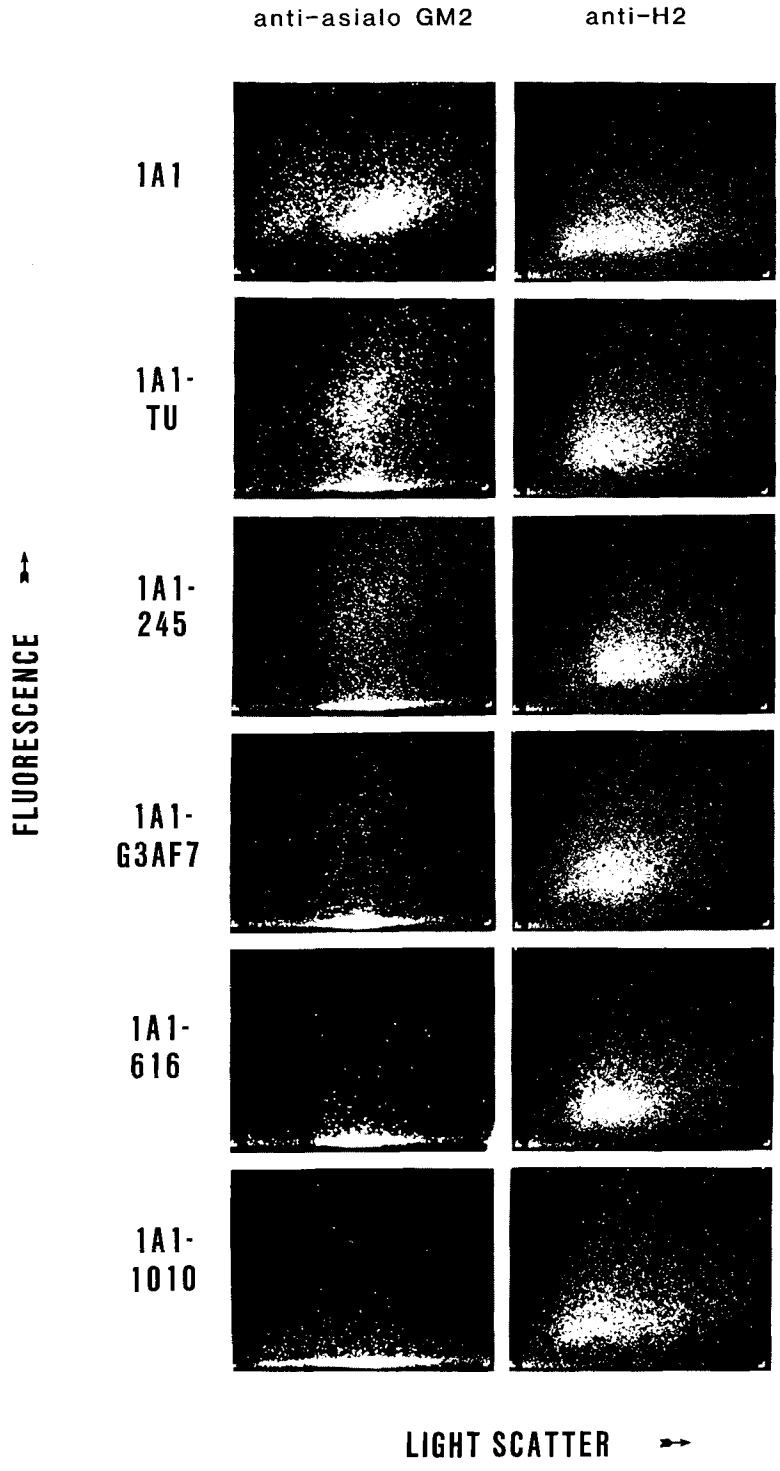

FIG. 2. Flow microfluorometry of L5178Y lymphoma cell lines with anti-asialo GM2 and anti-H-2^d. Lymphoma cell variants were labeled with IgM anti-asialo GM2 or mouse anti-H-2^d serum followed by fluoresceinated goat anti-mouse immunoglobulins and analyzed on the fluorescence-activated cell sorter as described in Materials and Methods. 1A1, cells used for challenge; 1A1-TU and 1A1-245, ascites tumor cells that arose in untreated mice; and 1A1-G3AF7, 1A1-616, and 1A1-1010, ascites tumor cells that arose in mice treated with IgG3 anti-asialo GM2. Abscissa, cell size as determined by light scatter; ordinate, fluorescence.

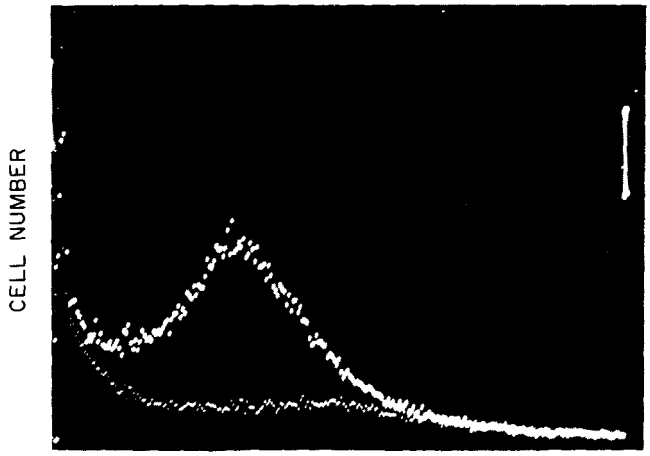

FIG. 3. Flow microfluorometric analysis of the stability of asialo GM2 expression on 1A1-TU cells. 1A1-TU cells were labeled with IgM anti-asialo GM2 followed by fluoresceinated goat anti-mouse immunoglobulins and sorted on a FACS-IV under sterile conditions. Asialo GM2-negative cells sorted from fluorescence channels 1–5 and positive cells obtained from channels 114–255 were grown in culture for 10 d and then reanalyzed by flow microfluorometry. Upper curve, asialo GM2-positive population, and lower curve, asialo GM2-negative population; ~50,000 cells were analyzed from each population.

for this assay rather than the IgG3 ascites because the former had a titer for complement-dependent cell lysis 10,000-fold higher than the latter. The results of lysis by anti-asialo GM2 plus complement (Table I) were consistent with the flow microfluorometry data. Cells from antibody-treated mice (1A1-G3AF7, 1A1-616, and 1A1-1010) were highly resistant, whereas lysis of the two cell lines from untreated mice (1A1-TU and 1A1-245) roughly corresponded to the percentage of positive cells determined by cell sorter analysis (Fig. 2). However, the level of antibody required to achieve maximal lysis suggested that the cells from untreated mice might be more resistant to the action of complement than 1A1 cells. A comparison of the variants with regard to the effect of anti-H-2^d serum revealed an approximately two- to fourfold difference in sensitivity to complement-mediated cytolysis (Table I). These differences appeared to be unrelated to the origin of each cell line; of the three cell lines from antibody-treated mice, one (1A1-G3AF7) was highly sensitive, whereas the other two (1A1-616 and 1A1-1010) were much more resistant. As H-2^d expression determined by flow cytometry was virtually identical among the variants (Fig. 2), these results suggested differences in susceptibility to complement action.

Susceptibility to NK Cell Attack. Variants of L5178Y cells have been shown to differ in their susceptibility to NK cell lysis (4, 8, 9). The possibility existed, therefore, that the cells that eventually arose in a few antibody-treated mice might have been able to proliferate because they could resist NK cell attack. Table II indicates that these cell lines were only slightly less sensitive to NK cell lysis than the highly sensitive 1A1 cells. For comparison, data are included in Table II for the NK-resistant variant of L5178Y designated clone 27AV (8). The data do not support a role for NK cells in the selection of ascites tumor cells that survived anti-asialo GM2 serotherapy.

Resistance of Emergent Cells to Serotherapy. The data described above suggest that the ascites cells that eventually arose in mice after 1A1 cell challenge and anti-asialo GM2

TABLE I

*Complement-mediated Cytotoxicity of L5178Y Cell Variants by Anti-Asialo GM2 Ascites and Anti-H-2^d Serum**

L5178Y variant	Percent specific cytolysis at ascites dilution				
Monoclonal IgM anti-asialo GM2 ascites fluid					
	10^{-6}	10^{-5}	10^{-4}	10^{-3}	10^{-2}
1A1	11.2	50.8	88.5	90.9	97.3
1A1-TU	0	1.6	7.0	22.5	34.5
1A1-245	2.3	2.5	8.7	19.4	26.9
1A1-G3AF7	1.4	0.3	1.2	3.4	3.4
1A1-616	1.3	0.8	0.4	0.4	0
1A1-1010	0.2	0	0	0	0
Mouse anti-H2^d serum (NIH serum D-13)					
L5178Y variant	Percent specific cytolysis at serum dilution				
	1:640	1:320	1:160	1:80	1:40
1A1	43.6	82.7	91.8	95.9	97.2
1A1-TU	15.3	52.7	75.3	83.2	86.1
1A1-245	11.1	54.3	80.5	84.9	88.1
1A1-G3AF7	49.2	75.0	84.2	86.0	89.3
1A1-616	2.8	11.9	34.2	55.5	69.5
1A1-1010	4.2	30.9	59.4	77.7	84.9

* Microcytotoxicity assays were performed on ^{51}Cr-labeled target cells as described in Materials and Methods. Percent specific cytolysis in the presence of antibody alone or antibody plus heated guinea pig serum (as the complement source) was <2% in all cases.

TABLE II

*Susceptibility to NK Cells of L5178Y Lymphoma Cell Variants**

L5178Y variant	Percent specific cytolysis by NK cells at effector/target cell ratio			
	12:1	25:1	50:1	100:1
Experiment 1				
1A1	15.2	25.7	38.7	52.5
1A1-245	10.7	17.9	29.9	43.3
1A1-G3AF7	13.8	23.4	37.7	47.7
Clone 27AV	3.1	3.9	4.4	5.6
Experiment 2				
1A1	11.0	18.3	29.9	43.2
1A1-TU	8.2	15.6	24.4	38.6
1A1-616	8.2	15.2	26.1	37.9
1A1-1010	7.3	12.6	19.0	31.7

* Microcytotoxicity assays were performed on ^{51}Cr-labeled target cells as described in Materials and Methods.

treatment may have been able to withstand serotherapy because they were deficient in asialo GM2. To directly test this point, mice were challenged with 10^5 1A1-G3AF7 cells and then treated with IgG3 anti-asialo GM2 ascites fluid. Serotherapy had no

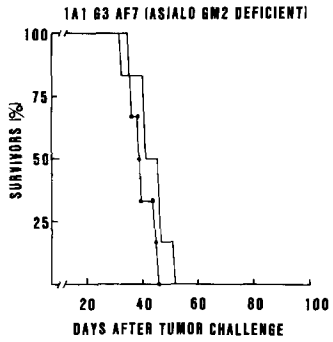

FIG. 4. Failure of serotherapy protocol to protect against challenge with asialo GM2-deficient lymphoma cells. L5178Y lymphoma subclone 1A1-G3AF7 cells arose in a mouse 76 d after challenge with subclone 1A1 cells and treatment with IgG3 anti-asialo GM2. CD2·F_1 mice were challenged with 10^5 1A1-G3AF7 cells intraperitoneally on day 0. One group was treated intraperitoneally on days 1, 3, 7, and 10 with 0.1 ml ascites fluid containing IgG3 anti-asialo GM2 (●); untreated control group (—). Each group contained six mice.

effect on survival of these mice (Fig. 4), indicating that these cells were able to proliferate despite the immune pressures present in antibody-treated mice.

Discussion

Considerable effort is presently being devoted to the production of monoclonal antibodies directed against human tumor-associated cell surface markers. Several of these antibodies have been found to react with specific glycolipids (10–13). These antibodies may prove useful not only for cancer diagnosis but also therapy (1). An obvious limitation of such specific serotherapy would be the appearance of antigen-negative tumor cells, which could avoid treatment. The generation of such variants is a predictable and essential element in the phenomenon of tumor progression (14) by which cancer cells are able to evade any selective pressure. Thus, it was not surprising that we detected asialo GM2 deficient variants arising in a few of the mice that had been challenged with 1A1 cells and treated with monoclonal IgG3 anti-asialo GM2 (3). Similarly, Bernstein and Nowinski (15) found Thy-1.1-negative leukemia cell variants in mice that had been challenged with Thy-1.1-positive leukemia cells and treated with anti-Thy-1.1-specific monoclonal antibodies.

The unexpected finding of the present study was the appearance of asialo GM2-deficient tumor cells in mice that had not been treated with anti-asialo GM2 (1A1-TU and 1A1-245 cells; Fig. 2). Other groups have described alterations in tumor antigen expression that were due to shielding of cell surface determinants by other macromolecules (16, 17). This phenomenon is unlikely in our system because H-2 antigen display on the variants was unchanged (Fig. 2) and the chemical quantity of asialo GM2 on 1A1-TU cells, about half that of 1A1 cells (3), matched the percentage of cells that were reactive with anti-asialo GM2 (Fig. 2). During in vitro culture for >2 mo, >96% of 1A1 cells remained asialo GM2 positive as determined by cell sorter analysis. The expansion of asialo GM2-deficient cells during a similar period of time in vivo must have been the result of selective pressure. The selection process was specific, in that H-2 antigen expression was unchanged on the variants; thus, asialo GM2-negative cells may be the product of a host immune response against the glycolipid. We are currently attempting to detect such a response. Our preliminary

results indicate that whereas CD2·F_1 mice can be immunized to produce anti-1A1 cell antibodies, none of those antibodies is specific for asialo GM2 (unpublished observation). Alternatively, the selective pressure may have been directed against a complex of membrane determinants. It is unlikely that such a complex was the result of viral contamination, because the subclone 1A1 cells tested negative in a screening for 12 viral contaminants including Sendai virus (data not shown).

The tumor cells that emerged from both antibody-treated and untreated mice were only slightly less sensitive to NK cell attack than 1A1 cells (Table II), suggesting that resistance to NK lysis was not a critical element in the phenotype of the cells that survived. However, it is possible that NK cells may act in concert with more specific immune mechanisms to decrease the tumor load. In fact, Kawase et al. (9) recently reported that modulation of NK activity effected the tumorigenicity of other L5178Y subclones.

Weinhold et al. (18) described a tumor dormant state established in L5178Y immunized and challenged mice in which a prolonged period of clinical normalcy was followed by rapid tumor outgrowth. Cytotoxic T cells reactive with L5178Y cells were detectable during the early period of tumor dormancy. The tumor cells that eventually emerged expressed eightfold less serologically detectable tumor associated antigens than the original cells used for challenge. Our serotherapy system may have similar antitumor mechanisms at work that are directed against a battery of tumor-associated antigens, perhaps including asialo GM2. These host defenses, when combined with additional anti-tumor effects mediated by IgG3 anti-asialo GM2, may be sufficient to eradicate the tumor completely in the majority of mice.

Summary

Mice challenged with L5178Y lymphoma cells expressing high levels of the glycolipid asialo GM2 (gangliotriosylceramide) were protected from tumor growth by passive administration of a monoclonal antibody specific for the glycolipid; in a few antibody-treated mice, ascites cells eventually proliferated which contained a reduced chemical quantity of the glycolipid antigen (3). We now report that the cells emerging from antibody-treated mice had abnormal marker chromosomes identical to those in the cells used for challenge, indicating that the emergent cells were progeny of the challenge inoculum. Flow cytometric analysis revealed that asialo GM2 was undetectable on the surface of >95% of the tumor cells from antibody-treated mice, whereas surface display of H-2 determinants was unchanged from that of the cells used for challenge. Tumor cells arising in challenged but untreated mice consisted of a mixture of asialo GM2-positive and -negative cells, indicating the presence of selective pressures in these mice as well. None of the cells taken from tumor bearing mice differed significantly from the challenge cells in their susceptibility to natural killer cell attack, suggesting that resistance to natural killer cell lysis was not responsible for the proliferation of these cells in vivo.

When cells derived from an antibody-treated mouse were used to challenge mice, serotherapy with anti-asialo GM2 had no effect on mouse survival. These results suggest that serotherapy may complement a host anti-tumor response, from which only asialo GM2 deficient cells can escape.

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