ERADICATION OF ESTABLISHED HUMAN MELANOMA TUMORS IN NUDE MICE BY ANTIBODY-DIRECTED EFFECTOR CELLS

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Immune destruction of tumors is a complex process that involves a variety of effector mechanisms including cytotoxic T cells, macrophages, natural killer (NK)[1] cells, and humoral antibody. Recent advances in cell hybridization technology have focused attention on the potential usefulness of monoclonal antibodies (mAb) directed against tumor-associated antigens as a tool to partially suppress tumor growth and thereby enhance the effectiveness of immune effectors in eliminating tumors. The injection of monoclonal antibody directed to tumor-associated antigens together with the respective tumor cells into nude mice may result in suppression of tumor growth (1–5). In previous experiments (6) we were able to corroborate these results by using a monoclonal antibody specific for a chondroitin sulfate proteoglycan preferentially expressed on human melanoma cells. We found that tumor suppression was markedly enhanced when splenocytes, in addition to antibody, were injected (7). This obervation raised two important issues, one regarding the mechanism by which tumor growth is suppressed, the other, whether this therapy may be sufficiently powerful to eradicate progressively growing tumors. In the present report, we demonstrate that estblished human melanoma tumors can be eliminated by athymic (nude) mice by the infusion of a tumor-specific mAb and splenocytes with NK activity.

Materials and Methods

Tumor Cell Lines and In Vivo Tumor Rejection Assays. The human melanoma cell line M21, originally provided by Dr. D. Morton, University of California, Los Angeles, was carried in BALB/c nude mice as a subcutaneous tumor. Progressively growing tumors were removed and a cell suspension was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum for 10 d. These tissue culture–grown M21 cells were harvested and injected (7.5×10^6 cells per mouse) subcutaneously into 6–8-wk-old BALB/c athymic nude mice obtained from the nude mouse colony at the University of California, San Diego. In some experiments, C57BL/6 nude mice were used (Vivarium, Scripps Clinic and Research Foundation). 14 d after tumor inoculation, the tumors had a mean

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[1] *Abbreviations used in this paper:* ADCC, antibody-dependent cellular cytotoxicity; mAb, monoclonal antibody; NK, natural killer.

volume of 80–100 mm^3. At this point, mice received intravenous injections of either various doses of effector cells alone, 400 μg mAb 9.2.27 alone or a combination of both. Tumor size was measured twice weekly with a graduated calimeter, for a total time of 6 wk after tumor inoculation; animals with large tumors were sacrificed earlier. Tumor-free animals were observed a total of 8 wk to assure that no tumor growth occurred. Tumor volume was calculated by the formula $\pi d_1 d_2 d_3/2$, in which d is the diameter of the tumor.

Antibodies. mAb 9.2.27 originally produced in this laboratory was shown to be of IgG2a isotype and directed against a chondroitin sulfate proteoglycan preferentially expressed on human melanoma cells (8). IgG was purified from ascitic fluid by precipitation with 50% ammonium sulfate and protein A–Sepharose affinity chromatography (Sigma Chemical Co., St. Louis, MO) according to the method of Ey et al. (9). The following antibodies against NK cell markers were used: rabbit anti–asialo GM_1 antiserum (Wako Chemicals, Dallas, TX); anti-Qa5 and anti-Lyt-6.2 antisera, kindly provided by Dr. U. Hammerling (Sloan-Kettering Institute for Cancer Research, New York); and anti-NK1.1, kindly provided by Dr. S. Pollack, (University of Washington, Seattle). Nontoxic rabbit serum (Accurate Chemical & Scientific Corporation, Westbury, NY) served as a source of complement.

Treatment of Cells With Antibody and Complement. Mouse spleen cells (10^7/ml) were incubated for 30 min at room temperature with antisera diluted 1:20 to 1:50 in RPMI 1640 medium. The cells were centrifuged; the supernatant containing unbound antibody was discarded; and the cells resuspended in complement diluted 1:10 in RPMI 1640 medium and incubated at 37°C for 45 min. Cells were washed twice in ice-cold medium and stored on ice before assay for cytotoxicity.

In Vitro Cytotoxicity Assays. Mouse mononuclear splenocytes, isolated by Hypaque Ficoll gradient centrifugation (LSM; Litton Bionetics Inc., Charleston, SC), were used as effector cells (7). BALB/c and C57BL/6 splenocytes served as normal effector cells whereas splenocytes from athymic BALB/c nu/nu mice were used as the source of T cell–deficient effector cells. Splenocytes from C57BL/6 mice with the homozygous beige mutation (Vivarium, Scripps Clinic and Research Foundation) were used as a source of NK-deficient effector cells. Cloned cells with NK activity (NKB61B10) were grown as described earlier (10). In vitro cytotoxicity of splenocytes against tumor cells was assayed in a ^{51}Cr release assay as previously described (7, 10). Briefly, tumor target cells were labeled with ^{51}Cr and plated in 96-well round-bottom tissue culture plates (Costar, Data Packaging, Cambridge, MA) at 10^4 (K562, Yac-1) or at 5×10^3 (M21) cells per well. Effector cells were added at various target/effector ratios. Replicate wells in triplicate were incubated at 37°C in CO_2 for 4 h (K562, Yac-1) or 16 h (M21). Cytolysis was calculated as: percent lysis = [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100.

To test for antibody-dependent, cell-mediated lysis (ADCC), we incubated tumor target cells with 50 μl per well of 9.2.27 antibody (ascites diluted 1:100) for 1 h at 37°C. The plates were centrifuged for 2 min, the supernatant fluids containing unbound antibody were discarded, and effector cells were added.

Results

Melanoma Growth in Nude Mice Is Influenced by a Component that Is Sensitive to Anti-asialoGM_1 Serum. In previous experiments (7), we had observed that the growth of M21 melanoma cells in nude mice can be suppressed by the simultaneous injection of mAb 9.2.27 specific for M21 and splenocytes at the time of tumor inoculation. We suspected that tumor suppression was caused by a cell-mediated mechanism and that the effector cells involved were NK cells, since this cell type expresses ADCC activity. To examine the possibility that M21 melanoma growth in nude mice is under the control of NK cells, we rendered BALB/c nude mice NK deficient by injection with anti–asialo GM_1 serum (11,

12). 24 h later, groups of untreated or anti-asialo GM_1–treated mice were inoculated subcutaneously with either 2.5×10^6 or 5×10^6 M21 cells. After 20 d, all mice that received a dose of 5×10^6 tumor cells showed tumor growth. The mean tumor volume in this anti-asialo GM_1–treated group was similar to the one in the untreated animals, i.e., 220 mm^3 vs. 190 mm^3 (Table I). A pronounced effect of the anti-asialo GM_1 treatment was seen, however, in animals that received 2.5×10^6 melanoma cells: All five mice that were treated with anti–asialo GM_1 developed tumors, whereas only one of five animals in the untreated group exhibited tumor growth. This result demonstrates that treatment with anti–asialo GM_1 greatly facilitates the growth of M21 melanoma cells in nude mice in the absence of injected antibody. Since in control experiments, we ascertained that injection of anti–asialo GM_1 very efficiently eliminates NK activity, this suggested that NK cells may indeed play a role in the suppression of melanoma growth in nude mice.

Progressively Growing M21 Tumors Are Eliminated in Nude Mice by Injection of mAb 9.2.27 and Mononuclear Splenocytes from Normal Mice. We previously observed (7) that M21 melanoma tumor growth in nude mice could be inhibited when mononuclear mouse splenocytes and mAb 9.2.27, either alone or in combination, were injected within 1 d after tumor cell inoculation. Based on these findings, we now examined whether a combination of mAb 9.2.27 and cell populations with NK activity could eliminate well established, growing M21 melanoma tumors in nude mice. Groups of BALB/c nude mice (Table II) with 2-wk-old subcutaneous melanoma (mean volume, 90 mm^3) were either left untreated (Table II, group 1), or were injected intravenously with mAb 9.2.27 IgG (Table II, group 2), BALB/c mononuclear splenocytes (Table II, group 3), or a combination of antibody and splenocytes (Table II, group 4). 4 wk after injection, 7 of 10 animals in the group that received both antibody and splenocytes (Table II, group 4) were tumor free. In contrast, all other mice exhibited large tumors, with the exception of one animal in the group that received splenocytes only (Table II, group 3). The mean volume of tumors in the group

TABLE I

Effect of Anti–Asialo GM_1 Injection on the Growth of Melanoma Tumors in BALB/c Nude Mice

Number of M21 tumor cells injected subcutaneously ($\times 10^6$)	Pretreatment with anti-asialo GM_1*	Number of mice with tumors‡	Mean tumor volume ± SE‡
			mm^3
2.5	+	5/5	185 ± 19
2.5	−	1/5	75
5	+	5/5	220 ± 29
5	−	5/5	190 ± 11

* 100 μl of anti–asialo GM_1 was injected intravenously 24 h before tumor injection.

‡ Data are for day 21 after tumor inoculation. Tumor-free mice were observed for a total of 35 d after inoculation.

TABLE II

Effect of Different Mononuclear Splenocytes and mAb 9.2.27 on Established Melanoma Tumors in Nude mice

Group	Injection of tumor-bearing mice*	Number of BALB/c nude mice with tumors per total‡	Mean tumor volume ± SE‡
			mm^3
1	Control (no injection)	9/9	1,359 ± 167
2	mAb 9.2.27	10/10	1,285 ± 151
3	BALB/c splenocytes§	9/10	605 ± 120
4	BALB/c splenocytes§ + mAb 9.2.27	3/10	127 ± 16
5	mAb 9.2.27	9/9	789 ± 130
6	BALB/c nude splenocytes§	8/9	504 ± 87
7	BALB/c nude splenocytes§ + mAb 9.2.27	3/9	149 ± 41
8	Control (no injection)	8/8	1,256 ± 174
9	BALB/c nude splenocytes§ (tumor-bearing mice)	7/8	960 ± 178
10	BALB/c nude splenocytes§ (tumor-bearing mice) + mAb 9.2.27	3/8	321 ± 92

* M21 tumors had a mean volume of 90 mm^3 14 d after inoculation in all experiments. mAb was injected at 400 μg per mouse in all experiments.

‡ Data are for the results obtained 28 d after antibody and cell injection, i.e., 42 d after tumor inoculation. Tumor-free animals were observed for a minimum of an additional 28 d.

§ 2×10^7 splenocytes were injected.

that received mAb 9.2.27 only (Table II, group 2) was nearly as great as that in the untreated control group. Tumors in the group that received effector cells only (Table II, group 3) were ~50% smaller. The mean volume of tumors in animals injected with splenocytes and antibody (Table II, group 4) was <10% of that of the control tumors 28 d after injection of splenocyte and antibody. Thus, the rejection of established and growing tumors in nude mice can be accomplished by the injection of antibody and mononuclear splenocytes from normal mice into the tumor-bearing host.

Cells Responsible for Antibody-induced Tumor Regression Are Present in Splenocytes of Tumor-free or Tumor-bearing Nude Mice. Since nude mice lack T cells, but the splenocytes from normal mice do not, it was possible that the effects observed were due to T cells. To examine this possibility, we injected tumor-bearing mice with mononuclear splenocytes from T cell–deficient nude mice. In groups 5, 6, and 7 (Table II), a combination of mAb 9.2.27 with splenocytes from BALB/c nude mice eliminated the growing tumors as effectively as the cells from normal BALB/c mice. Six of nine animals receiving antibody and splenocytes from nude mice (Table II, group 7) were tumor-free 4 wk after injection and the tumors in the remaining animals were much smaller than in the control group (149 ± 41 mm^3). In contrast, eight of nine mice treated with effector cells only (Table II, group 6) and all nine animals injected with antibody only (Table II, group 5) exhibited large tumors of 504 ± 87 and 789 ± 130 mm^3, respectively. This

suggests that the presence of T cells in normal splenocytes is not required for the elimination of tumors. Next, it was important to explore whether the effector cells responsible for tumor regression are also present in splenocytes of tumor-bearing nude mice or only in animals that are free of M21 tumors. In groups 8, 9, and 10 (Table II), cell populations able to suppress tumor growth were derived from either tumor-free or tumor-bearing nude mice. This suggests that tumor-bearing mice contain the effector cells responsible for tumor regression, although possibly too few of them to cause substantial tumor regression.

Effector Cells With NK Activity Are Responsible for Melanoma Tumor Regression in Nude Mice. Two lines of evidence suggest that NK cells are involved in tumor regression. First, growth of M21 tumor cells in nude mice is enhanced by in vivo treatment with anti-asialo GM_1 (Table I). Second, T cell-deficient nude mice reject the tumors in both the absence or presence of mAb 9.2.27 (Tables I and II). To explore this possibility further, we injected BALB/c nude mice with M21 tumor cells as before. After 14 d, when the tumors had a mean volume of 90 mm^3, mice were injected with 2×10^7 splenocytes, some of which had been treated with anti-asialo GM_1 and complement. Mice also received the usual dose of mAb 9.2.27. Only three of nine animals that received untreated splenocytes together with mAb 9.2.27 (Table III, group 2) showed very limited tumor growth after 4 wk. In contrast, all mice that received splenocytes treated with anti-asialo GM_1 and complement showed substantial tumor growth (Table III, group 3), essentially the same as that of the control group (Table III, group 1). These results indicate that the effector cells responsible for tumor regression carry the asialo GM_1 cell surface antigen, and suggest that they may be NK cells. To further test this conclusion, splenocytes from genetically NK-deficient mice carrying the homozygous beige mutation (13, 14) were also transplanted in tumor-bearing hosts. Specifically, C57BL/6 nude mice with progressively growing M21 tumors were injected with mAb 9.2.27 together with splenocytes from either normal C57BL/6 mice or C57BL/6 beige mice. None of the animals that received normal C57BL/6 splenocytes and antibody showed tumor growth after 4 wk (Table III, group 5), as was expected from the experiments with splenocytes from normal BALB/c mice (Table II, group 4). In contrast, animals that received mAb 9.2.27 and splenocytes from C57BL/6 beige mice experienced high tumor incidence (Table III, group 6), showing that the cell type responsible for tumor rejection is absent in beige mice and is therefore likely associated with NK activity. To further substantiate the conclusion that NK cells are responsible for tumor regression, we injected C57BL/6 nude mice that had established M21 tumors with the cloned NK cell line NKB61B10. In groups 7, 8, and 9 (Table III), a single dose of 2×10^6 NKB61B10 cells, i.e., a dose only 1/10th that of the splenocytes used in the other experiments, resulted in a strong suppression of tumor size (~70%), particularly in those mice that received antibody as well as NK cells. However, tumors were not completely eliminated, probably because of the uncommonly large size of the tumors at the onset of the experiment (mean volume, 185 mm^3).

M21 Melanoma Cells Are Lysed by Effector Cells with NK Activity in the Presence of mAb 9.2.27. The experiments so far presented point to the importance of the presence of both effector cells with NK activity and specific antibody for the

TABLE III

Evidence for NK Cells as Effectors in Suppression of Tumor Growth

Group	Injection of tumor-bearing mice	Number of nude mice with tumors per total	Strain	Mean tumor volume ± SE
				mm^3
1	Control (no injection)	9/9	BALB/c	930 ± 204
2	BALB/c nude splenocytes + mAb 9.2.27	3/9	BALB/c	149 ± 41
3	BALB/c nude splenocytes treated with anti-asialo GM1 + C + mAb 9.2.27	9/9	BALB/c	978 ± 126
4	mAb 9.2.27*	8/8	C57BL/6	741 ± 83
5	C57BL/6 splenocytes + mAb 9.2.27*	0/10	C57BL/6	—
6	C57BL/6 beige splenocytes + mAb 9.2.27*	8/9	C57BL/6	634 ± 292
7	Control (no injection)‡	7/7	C57BL/6	2,160 ± 292
8	NKB61B10‡	6/6	C57BL/6	1,060 ± 167
9	NKB61B10 + mAb 9.2.27‡	6/6	C57BL/6	610 ± 181

Data are given for day 28 after antibody and cell injection, i.e., 42 d after tumor inoculation. Tumor-free animals were observed for an additional 28 d.

* Mean tumor size at the onset of the experiments was 90 mm^3 at day 14 after tumor inoculation. 2 × 10^7 splenocytes were injected.

‡ Mean tumor size at the onset of the experiment was 185 mm^3 at day 14 after tumor inoculation. 2 × 10^6 NKB61B10 were injected.

elimination of an established tumor. Since it is known (15–19) that NK cells are able to perform ADCC, we examined whether M21 melanoma cells were lysed by mAb 9.2.27 and effector cells with NK activity. To this end, BALB/c mononuclear splenocytes, mixed with M21 melanoma target cells, were assayed for their cytolytic activity in the absence and presence of mAb 9.2.27 in ^{51}Cr release assays. We found that the effector cells caused 25% cytolysis in the absence of antibody (Fig. 1). After preincubation of the target cells with mAb 9.2.27, the cytotoxicity increased to 38%. The same effector cell population also showed lysis of the NK-sensitive YAC-1 target. The elimination of cells with NK activity by pretreatment of splenocytes with anti-asialo GM_1 serum and complement resulted in a marked reduction of cytotoxicity against M21 cells, both in the presence and absence of mAb 9.2.27. At the same time, the anti-asialo GM_1–treated effector cells were no longer able to lyse YAC-1 target cells, indicating that NK activity was indeed eliminated by this treatment. To further substantiate the conclusion that cells with NK activity were the effectors in this ADCC reaction, we used other antibodies that are specific for NK cells, i.e., anti-Qa5 and anti-NK1.1 (20–24). In addition, effector cells from NK-deficient C57BL/6 beige mice (13, 14) were tested. The data in Fig. 2 demonstrate that C57BL/6 mononuclear splenocytes mediated cytotoxic activity of the same magnitude, against M21 and YAC-1 target cells, as did BALB/c effector cells. Treatment of C57BL/6 effector cells with anti-Qa5 and anti-NK1.1, but not with anti-Lyt-6.2,

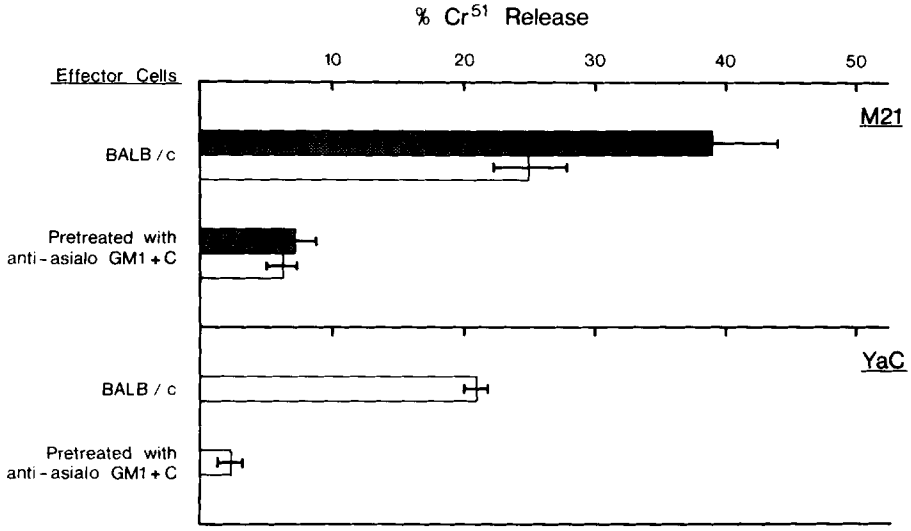

FIGURE 1. Effect of pretreatment of BALB/c mononuclear effector cells with anti-asialo GM_1 and complement on cytotoxic reactivity against M21 melanoma and Yac-1 target cells in ^{51}Cr release assays. (Shaded bar) M21 target cells preincubated with mAb 9.2.27; (open bar) effector cells alone.

resulted in a complete suppression of cytotoxicity of both YAC-1 and M21, regardless of the presence of mAb 9.2.27. In addition, C57BL/6 beige effector cells did not mediate an efficient cytotoxicity in these assays. These results strongly support the conclusion that cells with NK activity are indeed responsible for the antibody-dependent and antibody-independent cytolysis of M21 tumors.

Discussion

Our results show that the eradication of well established melanomas in athymic mice using tumor-specific antibody requires the simultaneous injection of a relatively large number of mononuclear splenocytes. Neither splenocytes nor antibody alone achieved significant tumor regression. The cells responsible for tumor elimination are probably cells with NK activity: they are present in splenocytes of T cell–deficient nude mice, and cloned NK cells are similarly able to suppress tumor growth. Moreover, the effector cells were absent in splenocytes of NK-deficient beige mice and could be eliminated by injection of mice with anti-asialo GM_1 antiserum, which is known to eliminate NK activity. Previous studies (20, 25–27) suggested that there may be a degree of heterogeneity within mouse NK cells, not only in cell surface markers but also in responsiveness to the regulatory influences of interferon and interleukin 2. It is therefore possible that the cells responsible for tumor regression are heterogeneous. Our observation that clone NKB61B10 was not quite as effective as normal splenocytes may support this possibility, especially since this clone very effectively prevents the rejection of bone marrow allografts and protects against radiation-induced leukemia (28). A more precise definition of the cell type responsible for tumor regression would have been possible using other NK-specific reagents, like anti-

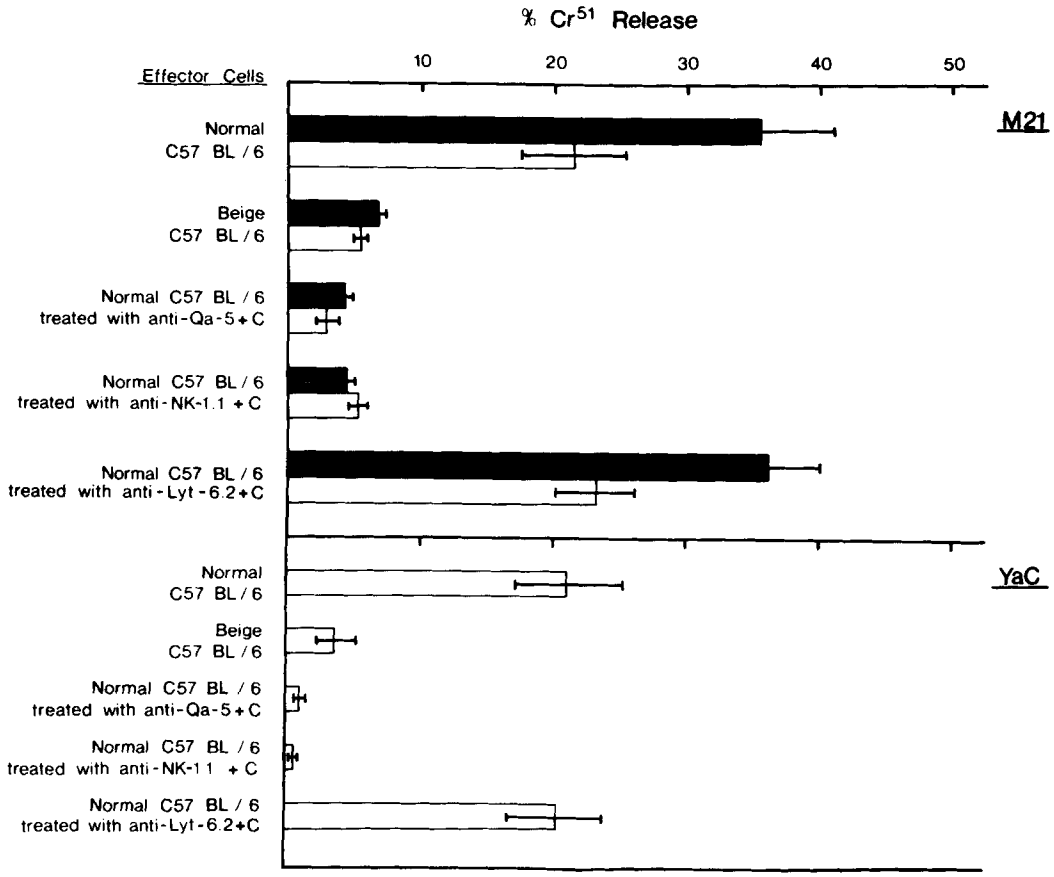

FIGURE 2. Comparison of cytotoxic effects against M21 human melanoma cells and Yac-1 target cells by mononuclear effector cells obtained from normal C57BL/6 mice, pretreated with anti-Qa5, anti-NK1.1, and anti-Lyt-6.2, as well as by effector cells obtained from C57BL/6 beige mice. (Shaded bar) M21 target cells preincubated with mAb 9.2.27; (open bar) effector cells alone.

Qa5 and anti-NK1.1 antisera, which are reactive with C57BL/6 NK cells but have little reactivity with other cell types, like T cells, natural cytotoxic cells, or monocytes (20, 21, 23, 27, 29). The limited amounts of these antisera available, however, precluded their in vivo use. We therefore demonstrated in vitro that both antibody-dependent and antibody-independent cell-mediated lysis of M21 melanoma targets is inhibited not only by anti-asialo GM_1 but also by anti-Qa5 and anti-NK1.1. The ability of anti-NK1.1 to eliminate NK cells in vivo and in vitro had been previously demonstrated by the work of Pollack (23, 24). Our results, therefore, strongly support the conclusion that both, antibody-dependent and antibody-independent in vitro lysis and in vivo elimination of tumor cells are functions of effector cells with NK activity. This conclusion is in full agreement with previous results demonstrating that cloned murine cell lines (10) with NK activity not only restored the ability of NK-deficient mice to suppress the growth of the murine melanoma B16, but also decreased the incidence of radiation-

induced leukemias (28). Moreover, the ability to reject bone marrow grafts is also restored by injection of cloned NK cells into NK-deficient mice. Interestingly, the specificity of marrow graft rejection depends on IgG antibody present in the graft recipient and specific for the bone marrow transplant (28, 30). The acute rejection of bone marrow grafts by NK cells is, therefore, due to an antibody-dependent cytotoxic reaction, very similar to the rejection of M21 melanoma tumors induced by mAb 9.2.27 and NK cells.

It is interesting that the injection of the tumor-specific mAb 9.2.27 into tumor-bearing nude mice is not sufficient to cause optimal tumor rejection, especially since such animals have a high level of NK activity. The explanation we favor is that the number of NK cells in the host is insufficient to cause tumor rejection. Another possibility is that host NK cells are blocked in their interaction with the mAb by circulating serum factors that inhibit their action; however, our experiments with spleen cells from tumor-bearing mice argue against this possibility. It may be important that antibody and effector cells be injected simultaneously so that the antibody can bind to the effectors, thereby "arming" them. This antibody–effector cell interaction may facilitate the targeting of effector cells to the tumor in a more efficient way. Previously (7), we noticed that the simultaneous injection of tumor cells and effector cells armed with antibody by conjugation with polyethylene glycol very efficiently suppresses in vivo tumor growth. Taken together, these results may suggest a novel approach for the treatment of tumors that makes use of two powerful tools, tumor-specific mAb and cytolytic effector lymphocytes.

Summary

The simultaneous injection of monoclonal antibody 9.2.27, directed against a chondroitin sulfate proteoglycan preferentially expressed on human melanoma cells, and 2×10^7 mononuclear splenocytes, eradicated established, progressively growing human melanoma tumors in nude mice. Neither splenocytes nor antibody alone achieved significant tumor regression. The cells responsible for tumor elimination are most likely natural killer (NK) cells: they are present in splenocytes of T cell–deficient nude mice, and cloned cells with NK activity are able to suppress tumor growth. Moreover, splenocytes treated with anti–asialo GM_1 and complement or harvested from NK-deficient C57BL/6 beige mice did not cause tumor rejection. Furthermore, treatment of BALB/c nude mice just before injection with anti–asialo GM_1 antiserum, which is known to eliminate NK activity in vivo, resulted in better tumor growth. In addition, evidence is presented that cells with NK activity are probably the effectors responsible for melanoma target cell lysis in vitro: Antibody-dependent and -independent cell-mediated lysis of M21 melanoma cells was suppressed when splenocytes were preincubated with complement and antibodies specific for cell surface antigens of NK cells, i.e., anti–asialo GM_1, anti-Qa5, and anti-NK1.1. Moreover, splenocytes of C57BL/6 beige mice were not able to lyse M21 cells in vitro. These results strongly support the conclusion that cells with NK activity are indeed responsible for the antibody-dependent destruction of M21 melanoma cells in vivo and in vitro.

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