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Monoclonal antibody-directed effector cells selectively lyse human melanoma cells *in vitro* and *in vivo*

(melanoma antibody/effector cell complex/immunotherapy)

GREGOR SCHULZ*, THOMAS F. BUMOL†, AND RALPH A. REISFELD*

*Department of Immunology, Scripps Clinic and Research Foundation, 10666 North Torrey Pines Road, La Jolla, California 92037; and †Lilly Research Laboratories, 307 East McCarty Street, Indianapolis, Indiana 46285

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ABSTRACT **Monoclonal antibody 9.2.27 (mAb 9.2.27) directed to a chondroitin sulfate proteoglycan on human melanoma cells was able to suppress tumor growth in athymic (*nu/nu*) mice more effectively when bound with polyethylene glycol to murine effector cells than when injected alone. These "armed" effector cells also proved more effective than the monoclonal antibody in eliciting antibody-dependent cellular cytotoxicity against human melanoma target cells *in vitro*.**

We have previously described the production of hybridomas secreting a monoclonal antibody 9.2.27 (mAb 9.2.27) which reacts with an antigenic structure expressed preferentially on the surface of human melanoma cells (1). In a series of additional experiments employing pulse–chase biosynthetic studies as well as biochemical and topographical analyses, we clearly identified the antigen recognized by mAb 9.2.27 as the core glycoprotein for a chondroitin sulfate proteoglycan expressed in the pericellular matrix of melanoma cells (2). In additional studies, we were able to show that mAb 9.2.27 *per se* could suppress the growth of human melanoma tumors in *nude* mice to an extent that was equally as great as when the same antibody was conjugated with diphtheria toxin A chain (3).

Several investigators have reported that monoclonal antibodies directed against malignant melanoma can mediate antibody-dependent cellular cytotoxicity (ADCC) *in vitro* (4, 5). Other studies suggested that "arming" effector cells with antibodies before incubation with target cells provides an alternative way to achieve specific cell killing *in vitro* by a mechanism termed directed ADCC (6, 7). These studies made use of anti-trinitrophenyl (TNP) "armed" effector cells and TNP-coated target cells or effectors armed with antibody to human chorionic gonadotropin and IEG-3 choriocarcinoma target cells. It was also demonstrated that this directed ADCC reaction was not inhibited by immune complexes as is the classical ADCC (6, 7).

The aim of our study was twofold: First, we wished to determine whether murine effector cells armed with a highly specific monoclonal antibody (mAb 9.2.27) against a well-defined antigen expressed on the surface of human melanoma cells are able to specifically kill these cells *in vitro* by a directed ADCC reaction. Second, we wanted to assess whether these antibody-coated or armed effector cells are able to achieve a more effective suppression of human melanoma tumor growth in *nude* mice than that obtained previously by injection of the mAb 9.2.27 *per se* (3).

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MATERIALS AND METHODS

Tumor Cell Lines. The M21 human melanoma cell line utilized for these studies was originally derived from a secondary metastasis of human melanoma by Donald Morton and colleagues at U.C.L.A. (8). In several experiments, we also used the M14 human melanoma cell line (9) and Panc-1 cells derived from a human cancer of the exocrine pancreas (10). The viability of all tumor cells used in the experiments exceeded 95% as measured by trypan blue exclusion.

Effector Cells. Spleens were removed from female 8- to 12-week old BALB/cByc mice that were bred and raised at the vivarium of Scripps Clinic and Research Foundation. After mincing, the spleen cells were washed twice in RPMI 1640 medium. Mononuclear cells were isolated by centrifugation (200 × *g*) through lymphocyte separation medium (LSM, Bionetics Laboratory Products, Kensington, MD). Cells of the interphase were washed twice in RPMI 1640 medium prior to use.

Monoclonal Antibodies. mAb 9.2.27, directed against the core glycoprotein of chondroitin sulfate proteoglycan on melanoma cells was developed in this laboratory (1, 2). Both KS1/4, a monoclonal antibody directed to lung adenocarcinoma and LC7/8, a monoclonal antibody directed against an antigen on human pancreatic cancer cells were also produced in this laboratory. Antibody IgG was isolated from ascites fluid by staphylococcal protein A-Sepharose affinity chromatography (Sigma) according to the method of Ey *et al.* (11).

Labeling of Tumor Cells. Tumor target cells were labeled with ^{51}Cr (sodium chromate at 1 mCi/ml, New England Nuclear; 1 Ci = 3.7×10^{10} Bq). Normally, 2×10^6 cells were incubated with 100 μCi of ^{51}Cr for 1 hr at 37°C in RPMI 1640 medium containing 10% fetal calf serum. The radiolabeled cells were then washed three times, resuspended in RPMI 1640 medium plus fetal calf serum and plated in 96-well tissue culture clusters with round bottoms (Costar, Cambridge, MA) at 5×10^3 cells per well.

"Arming" of Effector Cells. In general, we used the method described by Jones and Segal (6). As a modification of their procedure, we employed fetal calf serum instead of phthalate oils as a separation medium, because we found the oils difficult to remove and they often caused toxicity in mice. Briefly, effector cells (5×10^7/ml) were incubated at 4°C for 90 min with different concentrations of IgG and polyethylene glycol (PEG 20,000, Fisher). Removal of excess polyethylene glycol and unbound IgG was achieved by overlaying 2 ml of fetal calf serum with 500 μl of cell suspension followed by centrifugation at 200 × *g* for 10 min. The supernatant was removed and the cell sediment was washed again and then resuspended in RPMI 1640

Abbreviations: mAb, monoclonal antibody; ADCC, antibody-dependent cellular cytotoxicity; K, killer; NK, natural killer.

medium, supplemented with 10 mM Hepes (Sigma), 200 mM glutamine (GIBCO), and 10% heat-inactivated fetal calf serum.

Assay of Directed ADCC. Armed effector cells were added to labeled tumor target cells at various effector-to-target cell ratios in a final volume of 200 μl in 96-well tissue culture clusters with round bottoms. All cultures were initiated in triplicate and incubated at 37°C in 5% CO_2 for different periods of time. Thereafter, the plates were centrifuged at 170 × *g* for 4 min. Then 100 μl of each supernatant was removed and the radioactivity was measured in a Packard gamma counter. The cell-mediated killing of the target cells was calculated as follows:

$$\% \text{ lysis} = \frac{\text{experimental cpm} - \text{spontaneous cpm}}{\text{maximal release cpm} - \text{spontaneous cpm}} \times 100.$$

In this equation, "spontaneous cpm" represents the radioactivity released from target cells in the absence of effector cells and maximal release was determined by treating the target cells with the detergent Nonidet P-40 at a concentration of 0.1%. To calculate specific killing, this value was corrected for natural killer (NK) activity, which was defined as target cell lysis in the presence of effector cells without antibody.

Assay of Classical ADCC. Target cells were incubated for 1 hr at 37°C with various IgG concentrations that corresponded to the amount of antibody bound to effector cells in the directed ADCC. After this incubation, plates were centrifuged at 45 × *g* for 2 min, and the supernatant fluids containing unbound antibody were discarded. Then effector cells were added at various target-to-effector ratios. Incubation time and calculation of specific release were exactly as in the directed ADCC.

Directed ADCC *in Vivo*. M21 cells (7.5×10^6) were implanted under the skin of 8- to 10-week-old athymic BALB/(*nu/nu*) mice obtained from the *nude* mouse colony facilities at the University of California at San Diego. Armed effector cells were injected into a tail vein. Tumor growth was determined by measuring three diameters (d_1, d_2, and d_3) of the subcutaneous tumors with graduated calipers and tumor volume was calculated by the formula $(\pi/2)(d_1 \cdot d_2 \cdot d_3)$ as given by Guiliani and Kaplan (12). The statistical significance of differences in mean tumor volume of each group was evaluated by Student's *t* test.

Fluorescein Labeling of Antibody. IgG in phosphate-buffered saline (1 mg/ml) was adjusted to pH 9.0 with 0.1 ml of a 0.5 M sodium borate buffer, pH 9.0, and then admixed with 100 μg of fluorescein isothiocyanate dissolved in 10 μl of *N,N*-dimethylformamide (Sigma). After an incubation for 3–4 hr at room temperature, the conjugate was dialyzed overnight against several changes of 200 ml each of phosphate-buffered saline, pH 7.2. The concentration of bound fluorescein was measured spectrophotometrically at 493 nm. About 5.5 μg of fluorescein was normally bound to 1 mg of IgG.

Analysis of Cellular Fluorescence. Effector cells were armed with different concentrations of fluorescein-labeled antibody as described above. These armed cells were stored at 37°C in a 5% CO_2 atmosphere. At different times, equal amounts of cells were removed, and washed twice in RPMI 1640 medium, and the cellular fluorescence was quantitated in a fluorescence-activated cell sorter (Becton Dickinson FACS-III).

RESULTS

Adherence of IgG to Effector Cells. Because monomeric IgG binds weakly to Fc receptor-bearing cells, polyethylene glycol was used to nonspecifically enhance binding of mAb 9.2.27 IgG to effector cells as previously described (6). When using 1 mg of fluorescein-labeled 9.2.27 IgG and 5×10^7 effector spleen cells at different times (5, 18, and 25 hr), we found 8% (vol/vol) polyethylene glycol to be optimal; 8 times more IgG remained cell associated than when no polyethylene glycol was used. IgG binding was relatively stable at this polyethylene glycol concentration, because even after 25 hr about 70% of the antibody remained fixed on the cell membrane.

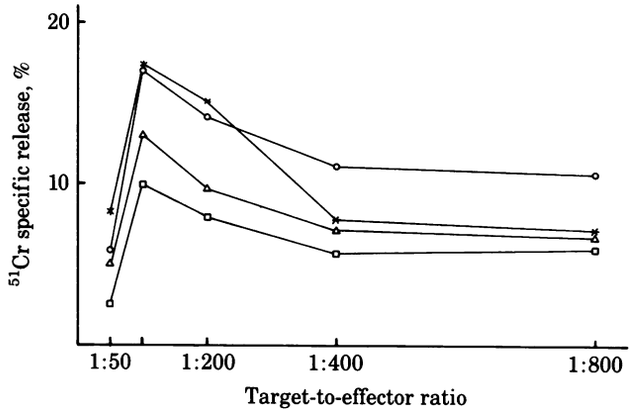

FIG. 1. Specific release in a ^{51}Cr assay using different target-to-effector cell ratios. Targets were M14 melanoma cells. Effector cells were mouse mononuclear cells armed with different concentrations of mAb 9.2.27: ×, 1.5 mg; ○, 1 mg; △, 0.75 mg; □, 0.5 mg; all are expressed as mg of IgG per 5×10^7 cells. Spontaneous release and release induced by NK cells are subtracted.

Optimization of Directed ADCC in the ^{51}Cr Release Assay. Fig. 1 depicts data obtained when effector cells were armed with different concentrations of mAb 9.2.27 (1.5, 1.0, 0.75, or 0.5 mg per 5×10^7 cells) and tested in a ^{51}Cr release assay against melanoma cells at various target-to-effector cell ratios. The concentration of polyethylene glycol was held constant at 8% in all these experiments. Preliminary studies (data not shown) indicated that the optimal incubation time for the ADCC reaction was 16 hr. The highest *specific* release was observed at a target-to-effector cell ratio of 1:100. Effector cells (5×10^7) armed with 1.5 mg and 1 mg of antibody, respectively, induced nearly the same killing, suggesting that optimal conditions were achieved. At target-to-effector cell ratios of 1:400 and 1:800 there was a decrease of specific release of ^{51}Cr, mainly because NK cell-triggered release is substantially greater than at the lower target-to-effector ratios.

Comparison of Directed and Classical ADCC *in Vitro*. The data depicted in Fig. 2 show that in five different experiments

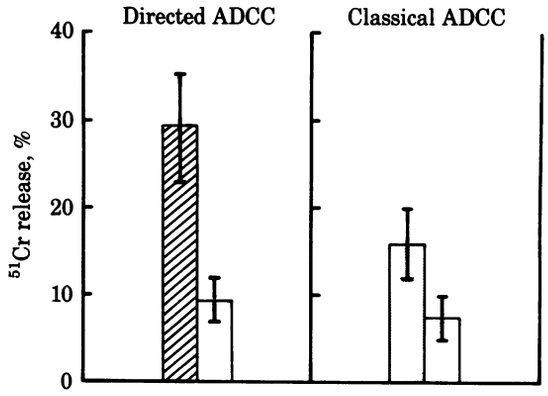

FIG. 2. Comparison of classical and directed ADCC reactions in a ^{51}Cr release assay at a target-to-effector ratio of 1:100. mAb 9.2.27 IgG concentration was 1 mg per 5×10^7 effector cells. Hatched bar, directed ADCC; stippled bar, classical ADCC; empty bars, effector cells without IgG; *Left*, with 8% polyethylene glycol; *Right*, without polyethylene glycol. Error bars indicate SD.

5×10^7 effector cells armed with 1 mg of 9.2.27 IgG (target-to-effector ratio = 1:100) induced a ^{51}Cr release from labeled human melanoma cells that was 29.3 ± 6.1% (mean ± SD) above the spontaneous release. Under identical conditions effector cells treated with 8% polyethylene glycol but not armed with mAb 9.2.27 IgG killed only 9.2 ± 2.7% of melanoma target cells. The difference was statistically highly significant at $P < 0.001$. When the same antibody IgG concentration at the same target-to-effector ratio was used, the mean specific ^{51}Cr release in the classical ADCC ($n = 4$) was only 16.1 ± 3.8%. Effector cells alone without IgG and polyethylene glycol induced a ^{51}Cr release of 7.6 ± 1.9%—i.e., the NK equivalent. The difference between directed and classical ADCC reactions was clearly significant at $P < 0.01$.

Specificity of Directed ADCC with mAb 9.2.27. To prove the specificity of melanoma cell killing, effector cells were armed with mAb 9.2.27 and tested in a ^{51}Cr release assay against the Panc-1 human pancreatic cancer cell line. As shown in Fig. 3 *Left*, effector cells armed with mAb 9.2.27 did not kill more Panc-1 cells in a 16-hr assay than did effectors not armed with this antibody—i.e., NK cells. However, when 5×10^7 effector cells were armed with 1 mg of mAb LC7/8 directed against a pancreatic associated antigen they did induce an additional specific release of over 20%.

In Fig. 3 *Right* it is shown that effector cells armed with mAb KS1/4 (1 mg per 5×10^7 cells) directed against lung cancer are not superior to NK cells in killing M21 melanoma target cells at a target-to-effector ratio of 1:100. As a positive control, we used effector cells armed with mAb 9.2.27 that induced a specific ^{51}Cr release of 20%. Essentially the same results were obtained when effector-to-target cell ratios of 1:50 and 1:400 were used (data not shown).

Suppression of Melanoma Tumor Growth in *nude* Mice by Effector Cells Armed with mAb. In an initial experiment designed to investigate the efficacy of armed effector cells in suppressing the growth of human melanoma tumors in *nude* mice, we implanted 7.5×10^6 M21 melanoma cells subcutaneously into 26 animals. Eight *nude* mice received seven intravenous injections (days 1, 2, 3, 5, 7, 9, and 12) of 10^7 effector cells armed with 200 μg of mAb 9.2.27 (group I). Another group of eight mice was injected with an equal number of effector cells and the same concentration of 9.2.27 IgG, but antibody and cells were mixed without addition of polyethylene glycol just prior to intravenous injection (group II). Ten animals did not receive any therapy (group III). As is evident from the data in Table 1, the mean tumor volume in the control animals (group III) was more than 3 times as large as that observed in both treatment groups I and II already at day 12, when the last injection was administered. The inhibition of tumor growth was similar in all animals of the two treatment groups. But whereas the tumors remained small and necrotic in group I animals even at days 25 and 32, the mean tumor volume was increasing again at the same time in all mice of group II. Thus, at day 32, the mean volumes of melanoma tumors were more than 3 times larger in those mice injected with a mixture of 9.2.27 IgG and effector cells than in animals that received the effector cell–antibody complex. Although both therapy regimens appeared quite effective, there was a decided difference: the tumors in the animals injected with the effector cell–antibody complex did not grow at all even after therapy was stopped, indicating that these tumors were biologically dead; however, under the same circumstances, growth of tumors in group II animals continued, though very slowly compared to control mice.

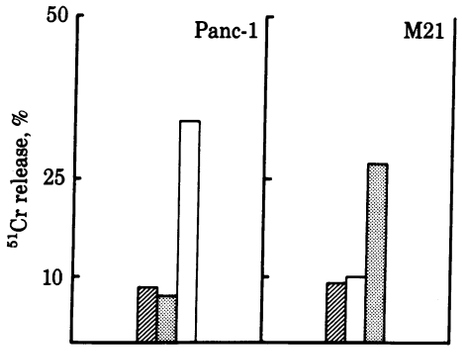

FIG. 3. Demonstration of specificity in a ^{51}Cr release assay by using different target and effector cells armed with various monoclonal antibodies. Target-to-effector ratios were 1:100. (*Left*) Targets are human pancreatic cancer cells (Panc-1). Hatched bar, effector cells are mouse mononuclear cells incubated with 8% polyethylene glycol but not with antibody (i.e., NK effect); stippled bar, mouse mononuclear cells armed with anti-melanoma mAb 9.2.27 (1 mg per 5×10^7 cells); open bar, mouse mononuclear cells armed with mAb LC7/8 (1 mg per 5×10^7 cells). (*Right*) Targets are human melanoma cells (M21). Hatched bar, effector cells are mouse mononuclear cells incubated with 8% polyethylene glycol but not with antibody (i.e., NK effect); stippled bar, mouse mononuclear cells armed with anti-melanoma antibody mAb 9.2.27 (1 mg per 5×10^7 cells); open bar, mouse mononuclear cells armed with anti-lung adenocarcinoma mAb KS1/4 (1 mg per 5×10^7 cells).

In another experiment, we tried to determine whether we could achieve the same efficiency of tumor suppression when binding less monoclonal antibody—i.e., 40 μg instead of 200 μg—to a smaller number of effector cells—i.e., 2×10^6 rather than 10^7. These armed effector cells were injected at days 1, 4, 8, 11, 15, and 18 (group I). As controls, we used *nude* mice that received only effector cells incubated with 8% polyethylene glycol (group II), mice that were injected only with mAb 9.2.27 IgG (group III), and finally, animals that received no therapy at all (group IV). All groups contained eight mice and all animals received 7.5×10^6 M21 melanoma cells subcutaneously at day 0. The data depicted in Fig. 4 indicate that the suppression of melanoma tumor growth by the effector cell–antibody complex is, even at this low concentration, similar to that observed with the higher dose therapy. Tumors of animals in group I became necrotic at day 15 and remained so until day 32—i.e., there was no observable increase in tumor volume. The inhibition of tumor growth in group I compared to that of untreated animals (group IV) was 93% at day 32. Inhibition of tumor growth was observed in group II (effector cells plus polyethylene glycol) and group III (mAb 9.2.27 alone) at 59% and 67%, respective to untreated control animals (group IV). While the inhibitory effect of mAb 9.2.27 alone agrees with the results obtained in a previous study (3), the inhibition of tumor growth achieved by polyethylene glycol-treated effector cells suggests that polyethylene glycol may activate the cytotoxic capacity of such cells. However, the mean volume of tumors in control groups II and III remained 5- to 6-fold greater than that of animals in group I, which received therapy with antibody bound to effector cells. The mean melanoma tumor volume in animals treated with armed effectors was significantly smaller than that of mice treated with mAb 9.2.27 IgG alone ($P < 0.01$), with effector cells only ($P < 0.01$), and untreated animals ($P < 0.001$).

In order to assess whether an immunity against a second tumor challenge developed, we reinjected 7.5×10^6 M21 cells subcutaneously into four mice in each treatment group 7 days after the last therapeutic injection. Within 1 week, not only all control animals but also all mice in the three therapy groups showed the appearance of a second tumor, suggesting that no prolonged immunity developed.

Table 1. Effect of armed effector cells on suppression of tumor growth *in vivo*

Time after injection of tumor cells, days	Group I		Group II		Group III
	Tumor vol, mm^3	Inhibition of tumor growth, %	Tumor vol, mm^3	Inhibition of tumor growth, %	Tumor vol, mm^3
12*	37 ± 13	73.4	44 ± 15	68.8	139 ± 43
18	57 ± 21	86.5	72 ± 23	83.0	423 ± 77
25	52 ± 18	90.5	112 ± 31	79.6	550 ± 93
32	47 ± 16	93.5	171 ± 45	76.3	720 ± 160

Results are presented as mean ± SEM.
* Last day of therapy.

DISCUSSION

The results of our experiments clearly indicate that the directed ADCC was at least twice as effective as the classical ADCC reaction in inducing specific tumor cell killing as measured by a ^{51}Cr release assay. In addition, only the directed ADCC reaction showed a statistically highly significant reactivity ($P < 0.001$) compared to that of NK cells. The relatively small (≈15%) specific release of ^{51}Cr observed in the classical ADCC reaction (target-to-effector ratio = 1:100) is not unusual because earlier studies by other investigators (4, 5) already indicated that, even at prolonged incubation times, a variety of monoclonal antibodies directed against human melanoma cells did not induce a specific release of ^{51}Cr greater than 15% at this same target-to-effector ratio. The relatively low efficiency of ADCC in melanoma is not surprising because it is known that cells of solid human tumors are far more resistant to cell-mediated cytotoxicity than are lymphoblastoid cells (13). Thus, in ADCC assays with lymphoblastoid target cells, only a very short incubation time (4 hr) and a lower target-to-effector ratio (1:25 or 1:50) is required to attain a very high specific release of ^{51}Cr. One reason for this difference may be that lymphoblastoid and solid tumor cells differ in their sensitivity to different subgroups of effector cells (14). Another reason for the relatively ineffective *in vitro* killing of melanoma cells by mouse spleen cells may be that mouse killer (K) cells are not as cytotoxic as K cells from man and other animals (15).

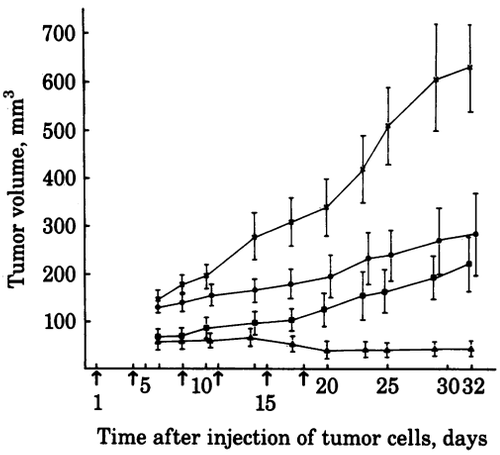

FIG. 4. Effect of monoclonal antibody and effector cells on suppression of tumor growth *in vivo*. The mean tumor volume (±SEM) was measured in *nude* mice that were inoculated subcutaneously with 7.5×10^6 M21 melanoma cells. Treated animals then received six intravenous injections each (arrows): ▲, animals treated with 2×10^6 effector cells armed with 40 μg of mAb 9.2.27 IgG (group I); ●, animals injected with 2×10^6 effector cells treated only with 8% polyethylene glycol (group II); ■, animals treated with 40 μg of mAb 9.2.27 IgG (group III). ×, Animals without any treatment (group IV).

Although the injection of effector cells treated only with polyethylene glycol resulted in some suppression of melanoma tumor growth, it was far smaller than that achieved by effector cells conjugated with mAb 9.2.27. This effect may have been induced by cytotoxic T cells because it was initially observed 10 days after the first injection, whereas antibody alone and antibody-effector cell conjugates showed an earlier effect on tumor growth. This observation may be explained by the fact that cytotoxic T cells are known to require several days for a primary response, whereas K cells and macrophages are able to kill tumor cells within hours (16). Treatment of effector cells with polyethylene glycol may actually have increased their ability to kill tumor cells, because preliminary experiments indicated a diminished effect on tumor growth suppression when effector cells not treated with polyethylene glycol were injected into tumor-bearing mice. The greater efficacy of the effector cell–antibody complex may be explained by the fact that the IgG fixed onto the surface of these cells by polyethylene glycol is less likely to dissociate *in vivo* and thus to bind to immune complexes or other serum blocking factors, both of which have been implicated in lowering the efficacy of tumor immunotherapy (17–19).

On the basis of a number of experiments, several hypotheses have been advanced concerning the mechanism(s) by which monoclonal antibodies are believed to effect the killing of tumor cells *in vivo*. Thus, Herlyn and Koprowski have demonstrated that growth suppression of human colorectal tumors in *nude* mice is mediated by macrophages with specific Fc receptors for antibodies of the IgG2a isotype (20). These investigators have also shown that complement is not required for this tumor cell destruction. The mAb 9.2.27 used in our experiments certainly satisfies these requirements because it is of IgG2a isotype and does not fix complement (1).

Although several investigators (21, 22) have shown that the interaction between macrophages and immunoglobulins is important for tumor killing, other cells, such as K cells, may also be involved in this process because they are known to kill malignant cells *in vivo*. In this regard, a common surface component of both macrophages and K cells are Fc receptors (16). Because the effector cells used in our experiments were unfractionated, we cannot identify the relevant cell types involved in this directed ADCC. At this time, we are unable to distinguish among possible mechanisms by which effector cells armed with mAb 9.2.27 IgG effect destruction of human melanoma tumors in *nude* mice. However, it appears likely that Fc receptors on macrophages, lymphocytes, or a combination of these and other factors are required for the tumoricidal effects we observed. Further studies are required to formulate a viable hypothesis for this potentially important process of tumor destruction.

To the best of our knowledge, no clinical application of polyethylene glycol-mediated arming of effector cells with monoclonal antibodies has been made. Sears *et al.* (23) have admin-

istered monoclonal antibodies together with a patient's monocytes in earlier trials of immunotherapy for colorectal cancer. No extraordinary effect was observed; however, the stability of a nonpolyethylene glycol-mediated formation of monoclonal antibody–effector cell complex is questionable and thus this concept remains to be tested under optimal conditions. Nevertheless, one can visualize that in patients with relatively low tumor load—e.g, after surgical resection, the patient's own effector cells armed with a specific monoclonal antibody or even a panel of such reagents could provide useful therapy or prophylaxis. For example, suitable effector cells could be collected at a time when the patient is still immunocompetent. Such cells could then be frozen (in dimethyl sulfoxide) until needed to be complexed with suitably specific monoclonal antibodies just prior to injection while using the patient's own serum to remove excess dimethyl sulfoxide and polyethylene glycol. Conceivably, such armed effector cells may under certain circumstances prove useful in preventing or at least slowing down recurrence of metastatic tumor growth.

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