IN VIVO TREATMENT WITH MONOCLONAL ANTIBODY 3.2.3 SELECTIVELY ELIMINATES NATURAL KILLER CELLS IN RATS

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Natural killer (NK) cells are large granular lymphocytes (LGL)[1] that express cytolytic activity in vitro against various tumor cells, virally infected cells, and some microorganisms (1–3). They do not productively rearrange TCR genes (4–6), do not express CD3 (7), and are capable of lysing target cells without major histocompatibility restriction (8, 9) or previous antigen sensitization (10, 11). Extensive evidence indicates that NK cells are responsible for rejection of certain implanted syngeneic tumors and inhibition of certain experimental tumor metastasis (12–15).

We have recently described an IgG1k mAb, termed 3.2.3, that recognizes a 60-kD disulfide-linked dimer composed of two 30-kD subunits expressed exclusively on fresh and IL-2-activated rat LGL/NK cells and polymorphonuclear cells (PMN) (16). mAb 3.2.3 can enhance cytolytic activity of LGL/NK cells against certain FcR^+ tumor target cells, by reverse antibody-dependent cellular cytotoxicity (ADCC) and also induces the release of BLT-esterase from IL-2-activated NK cells. These data suggest that mAb 3.2.3 recognizes a unique triggering structure present on fresh and IL-2-activated NK cells.

In this study we investigated the in vivo effects of mAb 3.2.3 in F344 rats. Our results show that intraperitoneal treatment of rats with mAb 3.2.3 for 1–3 d can completely and selectively eliminate NK and ADCC activity as well as LGL in the spleen and peripheral blood. Depletion of NK activity markedly decreased the survival of F344 rats injected intravenously with MADB106 mammary adenocarcinoma cells, confirming the important role NK cells play in controlling the metastatic spread of certain tumors.

Materials and Methods

Animals. Male Fischer 344 rats (75–100 g) were purchased from Taconic Farms, Inc. (Germantown, NY) and were housed in a specific pathogen-free animal facility at the Pittsburgh Cancer Institute for at least 10 d before use.

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[1] *Abbreviations used in this paper:* ADCC, antibody-dependent cellular cytotoxicity; LGL, large granular lymphocyte; PMN, polymorphonuclear cell; RPE, R-phycoerythrin.

Tumor Cells. The lysis of the NK-sensitive Moloney virus-induced mouse T cell lymphoma YAC-1 was used as an indicator of NK activity (17). The NK-resistant mastocytoma P815 (18) was used as the target in ADCC assays. The F344 mammary adenocarcinoma MADB106 (15) was injected into F344 rats for tumor survival experiments. All tumor cell lines were grown in RPMI 1640, 5% FCS, penicillin/streptomycin (complete medium, CM), and subcultured two to three times per week.

Preparation of Lymphoid Cells. Spleens were aseptically removed and single cell suspensions were prepared in RPMI 1640 with 5% FCS. Peripheral blood was obtained by cardiac puncture using heparinized syringes. Mononuclear cells were obtained after centrifugation on Ficoll-Hypaque gradients (density 1.077) at 300 *g* (for spleen cells) or 400 *g* (for PBMC) for 20 min. All lymphoid cells were washed twice in RPMI 1640 with 5% FCS and counted before use. Viabilities were routinely >97% by trypan blue exclusion. In some experiments, spleen mononuclear cells were passed over nylon-wool columns to remove monocytes/macrophages and B cells (19).

Cytotoxicity Assay. Cytotoxicity was measured in a standard 4-h ^{51}Cr-release assay using 96-well, round-bottomed microplates (Costar, Cambridge, MA). The target cells were labeled with 100 μCi of $Na_2{}^{51}CrO_4$ per 2×10^6 cells, washed, and seeded at 5×10^3 cells/well. Suspensions of effector cells were then added to triplicate wells to give various E/T ratios in a final volume of 200 μl. After an incubation at 37°C for 4 h, 100 μl of supernatant was removed from each well and was counted in a gamma counter to determine experimental release. Spontaneous release was obtained from wells receiving target cells and medium only, and total release was obtained from wells receiving 1% Triton X-100. Percent cytotoxicity was calculated by the following formula: Percent cytotoxicity = 100 × [(experimental release-spontaneous release)/(total release-spontaneous release)]. Assays of ADCC were performed as described above, with additional pretreatment of the P815 targets with 10 μl of rat anti-P815 serum for 30 min.

Proliferative Response to Con A. Spleen cells from control or NK-depleted rats were cultured in triplicate in 0.2 ml complete medium (CM) at a concentration of 1×10^5 cells/well in the presence or absence of Con A (10 μg/ml) in 96-well flat-bottomed microplates. After 72 h of incubation at 37°C in 5% CO_2/95% air, the cells were pulsed with 1 μCi of [^{3}H]thymidine (New England Nuclear, Boston, MA) for 12 h and subsequently harvested with a MASH II harvester for measurement of their radioactivity in a scintillation counter.

Antibodies. The following anti-rat mAbs were used: OX8 (CD8), OX19 (CD5), OX34 (CD2), OX41 (macrophages, dendritic cells), and W3/25 (CD4) were all purchased from Bioproducts for Science (Indianapolis, IN). ART 18 (which identifies the low affinity IL-2 receptor CD25) was a gift from T. Diamantstein (Freie Universität Berlin, Berlin, FRG). R73 (which identifies the rat TCR-α/β) was kindly provided by T. Hünig (Ludwig Maximilians University, Munich, FRG) (20). Rabbit anti-asialo GM_1 serum was obtained from WAKO Chemical (Dallas, TX). mAb 3.2.3 was produced as previously described (16). IgG antibody was purified from ascites fluid by affinity chromatography over protein A-Sepharose and was shown to be 98% pure by SDS-PAGE analysis. When used for FACS analysis, directly labeled FITC-F(ab')$_2$ fragments were used. For fluorescence staining each of the various antibodies was used at an optimal dilution based on preliminary dose-response titrations. All second-step antibodies were RPE- or FITC-labeled F(ab')$_2$ fragments of goat antibody against the primary antibody (Cappel Laboratories, Malvern, PA).

Flow Cytometry. For surface marker analysis, 2.5×10^5 cells in 0.1 ml of staining buffer (0.1% sodium azide and 2% FCS in PBS, pH 7.3) were incubated with various antibodies for 30 min at 4°C. The cells were washed twice with staining buffer and resuspended in FITC-labeled second antibody. After 30 min at 4°C, the cells were again washed twice with staining buffer, resuspended in 1% paraformaldehyde, and analyzed for fluorescence staining on a FACStar flow cytometer (Becton Dickinson & Co., Mountain View, CA).

Evaluation of Cell Morphology. Morphologic differentials were determined microscopically from Giemsa-stained cytocentrifuge slides or Giemsa/Wright-stained blood smears. At least 200 cells were examined by two independent observers for each slide.

Treatment of Rats. F344 rats were injected intraperitoneally with varying doses of affinity-purified mAb 3.2.3 or mAb 3.2.3 ascites (containing a total protein concentration of 10 mg/ml

of which 3 mg/ml was shown to be monoclonal IgG1) in 1 ml of HBSS once a day for 1-3 d. Control animals received injections of ascites fluid containing irrelevant IgG1 antibody or purified irrelevant IgG1 antibody each diluted in HBSS. In tumor challenge experiments, animals were treated with one dose of 100 μl of mAb 3.2.3 ascites intraperitoneally and 3 d later were injected with 1.5×10^6 MADB106 cells intravenously or 5×10^6 MADB106 cells subcutaneously. In one experiment animals were pretreated for three consecutive days with 50 μg/ml affinity-purified 3.2.3 intraperitoneally and 1 d later were injected intravenously with 0.5×10^6 MADB106 cells. In vivo growth of subcutaneous tumors was measured over a period of 30 d with Vernier calipers measuring 2 diameters at right angles.

Statistics. For survival experiments, Kaplan-Meier curves were drawn and the differences in survival between the various groups was assessed using the generalized Savage (Mantel-Cox) log-rank test. For statistical analysis of blood differential counts the Mann-Whitney and the Student's *t*-test were used. All other statistical analysis was performed using the Student's *t*-test.

Results

Effect of In Vivo Treatment of F344 Rats with mAb 3.2.3 on NK Activity and ADCC of Fresh Spleen Cells and PBMC. We treated F344 rats with 100 μl of mAb 3.2.3 ascites intraperitoneally once a day for 3 d. 1 d later (on day 4) both NK and ADCC activities of PBMC and spleen cells were determined. Table I shows that NK and ADCC activity of fresh PBMC and spleen cells were virtually eliminated after treatment with mAb 3.2.3. To study the dose dependence and kinetics of this effect, we treated rats with various amounts of mAb and measured NK activity of fresh spleen cells at different intervals after treatment. As shown in Fig. 1, treatment for 3 d with 100 μl mAb 3.2.3 ascites once a day could completely eliminate NK activity for >10 d. NK activity then returned to nearly control levels by day 19. The depression in NK activity was dose dependent as both the absolute level of depression as well as the duration of depression were dependent on the quantity of ascites administered.

We also studied the effects of a single dose treatment with either 50 μl or 100 μl

TABLE I

NK and ADCC Activities of Fresh Spleen Cells and PBMC from Untreated and 3.2.3-treated F344 Rats

Cells	Treatment of rats	NK-activity (cytotoxicity vs. YAC-1) 100:1*	NK-activity (cytotoxicity vs. YAC-1) 50:1	ADCC (cytotoxicity vs. P815‡) Without anti-P815 serum	ADCC (cytotoxicity vs. P815‡) With anti-P815 serum
Spleen cells	Untreated	47 ± 3§	35 ± 2	3 ± 2	18 ± 3
	Control ascites	52 ± 8	42 ± 7	4 ± 0.5	17 ± 3
	3.2.3 ascites	1 ± 2	0 ± 2	0 ± 4	0 ± 1
PBMC	Untreated	22 ± 4	20 ± 3	0 ± 0	18 ± 2
	Control ascites	20 ± 3	16 ± 2	1 ± 1	16 ± 1
	3.2.3 ascites	4 ± 1.5	4 ± 2	0 ± 0.5	1 ± 1.5

Rats were treated for 3 d with 100 μl of mAb 3.2.3 ascites or control ascites once a day i.p. On day 4, mononuclear cells were obtained and tested for NK and ADCC activity in 4-h ^{51}Cr-release assays.

* Effector/target ratio.

‡ Effector/target ratio of 50:1.

§ Mean percentages ± SD of cytotoxicity from five rats in each group.

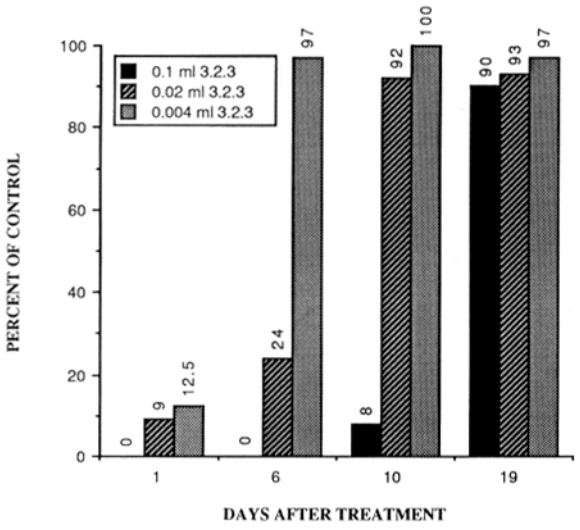

FIGURE 1. NK activity in F344 rats treated with varying doses of mAb 3.2.3 ascites for 3 d (one injection/day, i.p.). NK activity was determined as lytic activity of fresh spleen cells from 3.2.3-treated rats against YAC-1 targets at an E/T ratio of 50:1 and compared with NK activity of control rats treated with irrelevant ascites. Each column represents mean NK-activity of two rats expressed as percentage of the mean NK activity of two control rats. NK activity of control rats was 26 ± 9%.

mAb 3.2.3 ascites and found that depletion of NK activity could last up to 4 d after a single treatment with 50 μl mAb and up to 11 d after a single treatment with 100 μl mAb (Fig. 2).

To rule out that the NK depletion in vivo was caused by a nonspecific effect of the mAb 3.2.3 ascites fluid, we treated rats with different concentrations of affinity-purified antibody. Fig. 3 shows that even treatment with a dose as low as 0.05 mg of affinity-purified 3.2.3 IgG could completely deplete splenic NK and ADCC activity.

Cell Surface Phenotype and Percentage of LGL in Fresh Spleen Cells from 3.2.3-treated Rats. The selective depletion of NK activity in 3.2.3-treated rats correlated with an altered expression of cell surface markers associated with NK cells. Table II shows a decreased expression of CD2 (present on T cells and NK cells), CD8 (present on T cytotoxic/suppressor cells and NK cells), asialo GM_1 (present on NK cells and subpopulations of macrophages and cytotoxic T cells), and 3.2.3 (present on NK cells and PMN). Moreover, the percentage of LGL, as counted in Giemsa-stained cytospin preparations, was decreased from 4.3 ± 0.9 in untreated rats to 0.4 ± 0.4 in 3.2.3 treated rats.

The low number (6%) of $3.2.3^+$ cells that were still present after 3.2.3 treatment were found to express the 3.2.3 antigen only dimly (Fig. 4). Two-color FACS analysis showed that half of these 3.2.3(dim+) cells coexpressed CD5, indicating a T cell origin. Spleen cells from normal rats also contain a subpopulation of $CD5^+$/3.2.3(dim+) cells (~6%), and we found that these cells are $CD8^+$, $CD2^+$ and TCR-α/β^+ T cells that are not capable of lysing NK-sensitive targets or of ADCC even after 5 d of culture with IL-2 (manuscript in preparation). Interestingly, Fig. 5 also shows a small subpopulation (3.0%) of $CD5^-$/3.2.3(dim+) cells, which is present in spleen cells from 3.2.3-treated rats, but not in spleen cells from normal rats. Further analysis of these $CD5^-$/3.2.3(dim+) cells in 3.2.3-depleted rats showed that these cells are $CD2^+$, $CD8^+$ (~50%), $CD4^-$, TCR-α/β^-, and display less cytolytic activity against NK-sensitive targets than $CD5^-$/3.2.3 (bright+) cells (data not shown). This suggests that these $CD5^-$/3.2.3(dim+) cells, which were only found

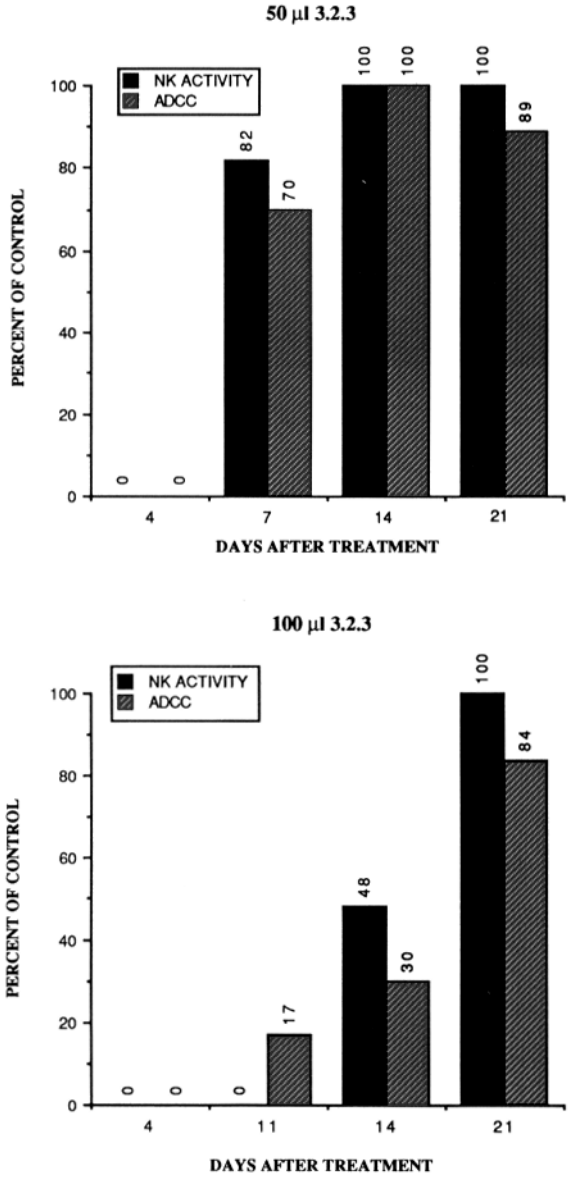

FIGURE 2. NK and ADCC activity in F344 rats treated with one dose of 50 or 100 µl i.p. of mAb 3.2.3 ascites. Each column represents mean splenic NK activity (E/T ratio 50:1) or ADCC (E/T ratio 25:1) of two rats expressed as percentages of the mean NK or ADCC activity of two control rats. NK activity of control rats was 23 ± 7% and ADCC was 21 ± 6%.

in 3.2.3-treated rats, could be either immature NK cells or functionally impaired NK cells. Table II also shows that the total number of T cells ($OX19^+$ as well as TCR-α/β^+) was not affected by the treatment with 3.2.3.

T Cell Function and Numbers of PMN Are Not Affected by Treatment of F344 Rats with mAb 3.2.3 Ascites. Rats were treated for 3 d with 100 µl of mAb 3.2.3 ascites once a day. 1 d after treatment, spleen cells were cultured with 10 µg/ml Con A. After 72 h proliferation was determined in a 12-h [^{3}H]thymidine incorporation assay. Table III shows that T cell function, as measured by Con A stimulation in vitro, was not affected by treatment with mAb 3.2.3 ascites in vivo.

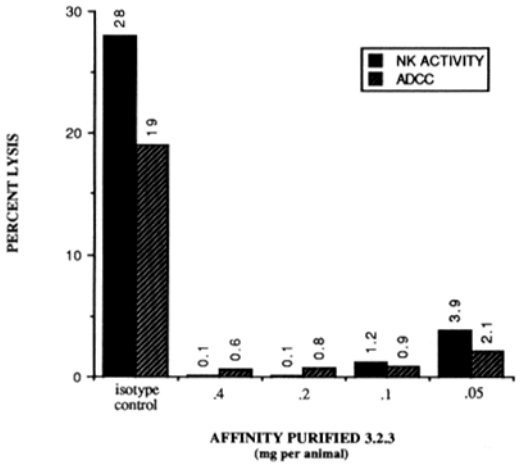

FIGURE 3. NK and ADCC activity in F344 rats treated with varying doses of affinity-purified mAb 3.2.3 or purified irrelevant IgG for two consecutive days. Splenic NK activity (E/T ratio 50:1) and ADCC (E/T ratio 50:1) was determined 2 days later (day 4). Each column represents mean NK or ADCC activity of two rats.

We also performed blood differential analysis on Giemsa-Wright-stained peripheral blood smears to determine if PMN, which express low levels of the antigen identified by mAb 3.2.3, are affected by treatment with mAb 3.2.3 ascites in vivo. Table IV shows that no significant differences in percentages or absolute numbers of PMNs, lymphocytes, or monocytes were noted after treatment with mAb 3.2.3. Also the percentage of immature PMNs with band-shaped nuclei was not changed.

TABLE II

Phenotype and Percentage of LGL in Fresh Spleen Cells from Untreated and 3.2.3-treated F344 Rats

Surface markers	CD equivalent	Percentage of positive spleen cells from: Control rats	3.2.3-Treated rats
OX19	CD5	81 ± 1*	84 ± 2
W3/25	CD4	41 ± 1	40 ± 1
OX8	CD8	49 ± 2	41 ± 1
OX34	CD2	89 ± 3	82 ± 1
R73	TCR-α/β	75 ± 2	74 ± 3
ART 18	CD25	4 ± 2	4 ± 1
OX41	Macrophages	3 ± 2	3 ± 2
Asialo GM_1	NK cells, subpopulations of macrophages, and cytotoxic T cells	74 ± 5	63 ± 2
3.2.3 (bright +)	NK cells	12 ± 1	0 ± 0
3.2.3 (dim +)	PMN and a subset of $CD8^+$ T cells	6 ± 2	6 ± 1
LGL‡		4.3 ± 0.9 (3-5)	0.4 ± 0.4 (0-0.8)

Rats were treated with a single injection of 100 μl of 3.2.3 ascites or irrelevant ascites. 3 d later, nylon wool-nonadherent spleen cells were analyzed for the expression of cell surface markers by flow cytometric analysis and for the percentage of LGL in Giemsa-stained cytospin preparations.

* Mean percentage ± SD from four rats in each group.

‡ Mean percentage ± SD from four rats in each group. Numbers in parentheses are ranges.

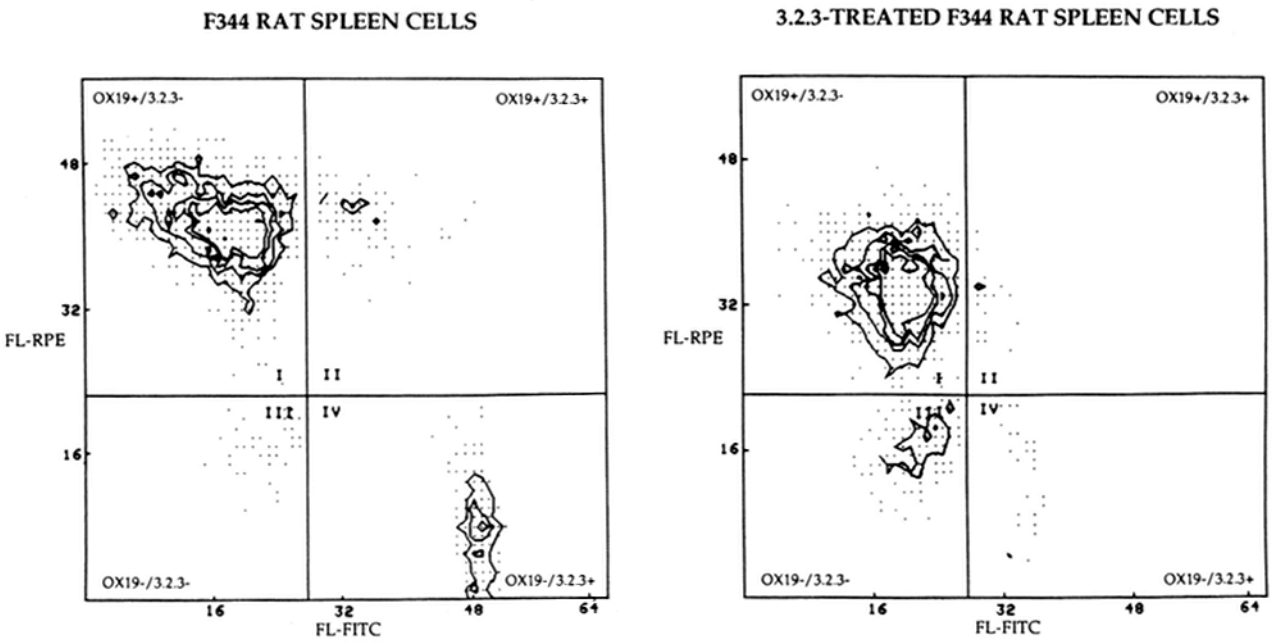

FIGURE 4. Two-color flow cytometric analysis of fresh spleen cells from control irrelevant ascites and 3.2.3-treated F344 rats with OX19 (CD5, FL-RPE) and 3.2.3 (FL-FITC). The green emission of FITC and the red emission of RPE are expressed on the *x*-axis and *y*-axis, respectively, on a linear scale in arbitrary units. Markers were set so that the percentages of PE^+ or $FITC^+$ cells in the isotype controls were <1%.

	Quadrant			
	I	II	III	IV
Cells	$OX19^+/3.2.3^-$	$OX19^+/3.2.3^+$	$OX19^-/3.2.3^-$	$OX19^-/3.2.3^+$
Control spleen cells	76.4	6.9	4.2	12.5
3.2.3-Treated spleen cells	82.5	2.4	12.1	3.0

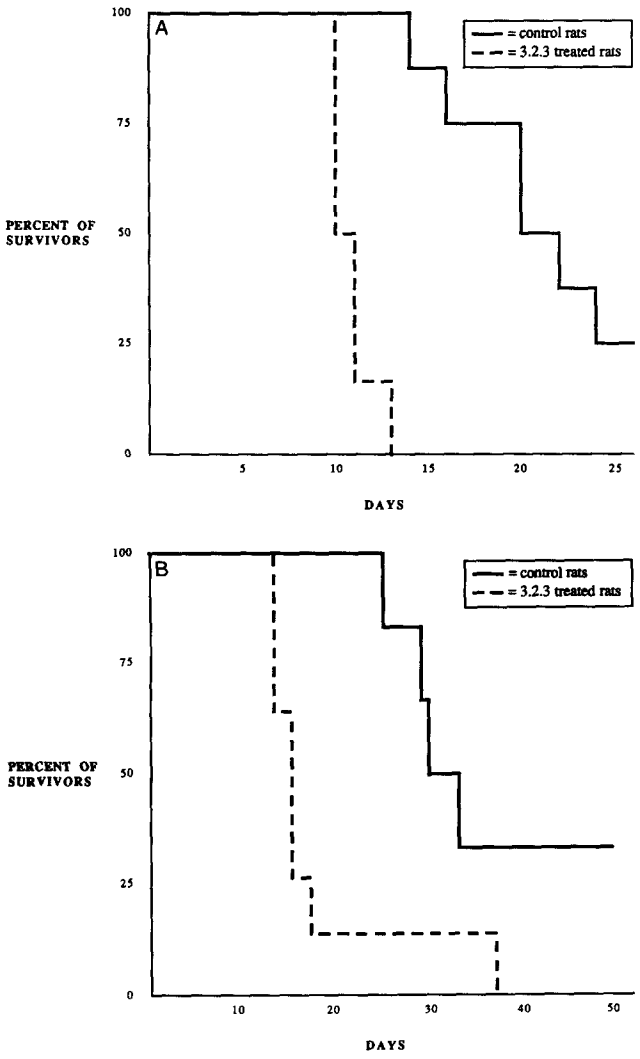

FIGURE 5. Survival of 3.2.3-treated or control IgG-treated F344 rats after injection of MAD-B106 cells i.v. (*A*) F344 rats were injected with 100 μl of mAb 3.2.3 ascites or irrelevant ascites in HBSS i.p. and received 1.5 × 10^6 tumor cells 3 d later. Groups consisted of six animals in the 3.2.3-treated and eight animals in the control group. Survival after tumor inoculation is shown with Kaplan-Meier curves. Statistical analysis with the Savage (Mantel Cox) log-rank test showed a significant difference in survival ($p < 0.0005$). Results are representative of two experiments with similar outcome. (*B*) F344 rats were pretreated for three consecutive days with 0.05 mg/ml affinity-purified 3.2.3 IgG or control IgG and received 0.5 × 10^6 tumor cells 1 d later. Groups consisted of six animals in both groups and the survival differed significantly (Savage (Mantel Cox) log-rank test; $p < 0.005$).

TABLE III

Proliferative Response to Con A of Fresh Spleen Cells from 3.2.3-treated F344 Rats

Exp.	Group*	Without ConA	With ConA
1	Control	3,404 ± 2,057‡	113,873 ± 11,222
	3.2.3-Treated	2,558 ± 1,836	124,122 ± 19,566
2	Control	3,708 ± 1,540	119,209 ± 11,159
	3.2.3-Treated	3,530 ± 1,554	163,859 ± 28,240

F344 rats were treated with 100 μl of 3.2.3 ascites or irrelevant ascites in 1 ml HBSS for three consecutive days. On day 4, spleens were removed and cells were cultured in triplicate at a concentration of 1 × 10^5 cells/well with or without 10 μg/ml Con A. After 72 h, cells were pulsed with 1 μCi of [^{3}H]thymidine for 12 h and subsequently harvested.

* Two rats/group.

‡ Mean ± SD of [^{3}H]thymidine uptake (cpm).

TABLE IV

Differential Cell Counts on Peripheral Blood from 3.2.3-treated F344 Rats

Treatment of rats	WBC ($\times 10^{-3}$/ml)	Polymorphonuclear cells			Lymphocytes	Monocytes
		Neutrophils	Eosinophils	Band-shaped nuclei		
Control IgG	9,895 ± 744*	13 ± 2.9‡	0.7 ± 0.2	2.1 ± 0.6	81 ± 2.1	3.1 ± 0.5
		(1,501 ± 416)	(72 ± 19)	(223 ± 68)	(7,290 ± 167)	(323 ± 75)
3.2.3	8,482 ± 181	15 ± 2.9	1.2 ± 0.2	2.0 ± 0.8	79 ± 4.0	3.1 ± 0.7
		(1,276 ± 264)	(106 ± 18)	(173 ± 67)	(6,666 ± 312)	(271 ± 63)

Rats were treated with 0.1 ml of mAb 3.2.3 ascites or irrelevant ascites (control) in 1 ml HBSS i.p. for three consecutive days (once a day). 1 day later (day 4) peripheral blood smears were prepared, Giemsa/Wright-stained, and analyzed for differential cell counts. No significant differences were found by statistical analysis with the Student's *t*-test or the Mann-Whitney test.

* Mean values ± SE of six rats in each group.

‡ Mean percentage ± SE and absolute number ± SE ($\times 10^{-3}$/ml) in brackets.

Effects of Treatment with mAb 3.2.3 on Subcutaneous Tumor Growth or Survival after Intravenous Injection of MADB106 Tumor Cells in F344 Rats. We studied the effects of NK depletion with mAb 3.2.3 on tumor growth and survival of the mammary adenocarcinoma MADB106 in F344 rats. Rats were treated with a single dose of 100 μl mAb 3.2.3 ascites and injected with 1.5×10^6 tumor cells intravenously 3 d later. Fig. 5 *A* shows the survival of 3.2.3 treated or control rats after tumor injection depicted as Kaplan-Meier curves. The 3.2.3-treated group had a significantly shorter survival than the control group (Savage (Mantel Cox) log-rank test; $p < 0.0005$).

Similar results were obtained when animals were pretreated with 0.05 mg/ml affinity-purified mAb 3.2.3 and injected 1 d later with 0.5×10^6 MADB106 cells intravenously (Fig. 5 *B*). In contrast, when 5×10^6 MADB106 cells were injected subcutaneously, no significant difference ($p > 0.05$) in tumor growth between the 3.2.3-treated and control groups was noted at any time over a 30-d period (Figure 6).

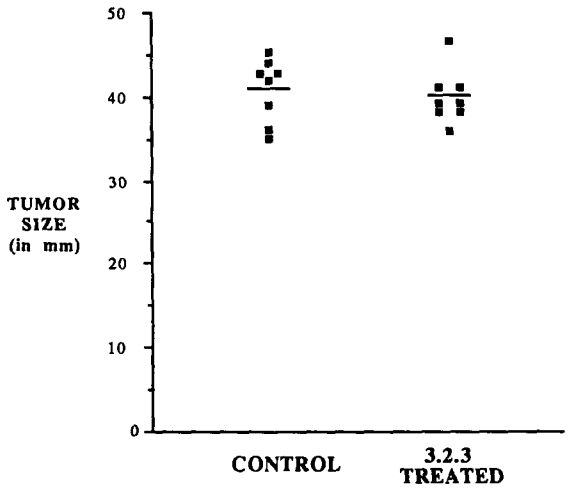

FIGURE 6. Subcutaneous tumor growth in 3.2.3 treated or control (HBSS treated) F344 rats after injection of 5×10^6 MADB106 cells s.c. Each group consisted of eight animals. Growth of s.c. tumors was measured over a 30-d period and presented as tumor size on day 17. Tumor sizes and means of each group are depicted. Statistical analysis using the Student's *t*-test showed no significant difference ($p > 0.05$) in s.c. tumor growth between control and 3.2.3-treated animals.

Discussion

In this study we have shown that in vivo treatment of rats with mAb 3.2.3, which recognizes a triggering structure present on NK cells, can completely and selectively eliminate NK and ADCC activities for >10 d. The NK depletion in vivo with mAb 3.2.3 was highly selective as T cell function, percentages of T cells, monocytes, or PMNs were not affected. Moreover, this is the first study to demonstrate that selective depletion of LGL/NK cells in vivo also eliminates ADCC function in vitro.

Chambers et al. (16) previously reported that mAb 3.2.3 did not block NK activity in vitro. The in vivo effects of mAb 3.2.3 are, therefore, most likely due to physical removal of NK cells either by complement-mediated lysis, ADCC or sequestration. The virtual absence of LGL in Giemsa-stained cytospin preparations, as well as the absence of 3.2.3(bright+) cells in the blood or spleen, supports this hypothesis. Barlozzari et al. (21) also found similar correlation between a decrease in NK activity and a decrease in the number of LGL after treatment with anti-asialo GM_1 antibody.

In concordance with the depletion of NK/ADCC function and LGL, we also found quantitatively decreased expression of NK-associated surface markers (CD2, CD8, asialo GM_1, and 3.2.3) on spleen cells from 3.2.3-treated rats but no changes in surface markers not expressed on NK cells (CD5, CD4, OX41, and TCR-α/β). The remaining 3.2.3(dim+) cells in 3.2.3-treated rats were found by two-color FACS analysis to contain $CD5^+$ as well as $CD5^-$ cells. The $CD5^+$ cells were unequivocally shown to be T cells as they also expressed the TCR-α/β. The $CD5^-$/3.2.3(dim+) cells present in 3.2.3-treated rats were found to coexpress CD2 and CD8 (~50%) but not CD5, CD4, or TCR-α/β. These cells showed less cytotoxicity against NK-sensitive targets than $CD5^-$/3.2.3 (bright+) cells and were not observed in normal F344 rats. They may represent either pre-NK cells, which appear in the spleens of rats depleted of active NK cells due to an unknown regulatory mechanism, or mature NK cells with decreased cytolytic function due to in vivo binding of 3.2.3, possibly leading to receptor modulation and functional impairment.

In addition to depletion of NK/ADCC activity, almost no LGL could be detected in Giemsa-stained cytospins of spleen cells or PBMC from 3.2.3-treated rats. The few remaining LGL after mAb 3.2.3 treatment were morphologically distinct from LGL observed in cytospin preparations from untreated rats. These cells were smaller in size and contained fewer and smaller granules (data not shown). These cells could either represent $CD5^-$/3.2.3(dim+) NK cells (as suggested above) or the $CD5^+$/3.2.3(dim+) T cells. Additional studies are currently in progress to determine these two possibilities.

The selective depletion of NK cells with mAb 3.2.3 resulted in a decreased survival after intravenous injection of MADB106 mammary adenocarcinoma cells. However, depletion of NK cells did not appear to influence the growth of MADB106 cells injected subcutaneously. Our findings with the growth of MADB106 intravenously or subcutaneously in NK-depleted animals support the results of other investigators that (a) NK cells are involved in the elimination of tumor cells in vivo (12–15), and (b) NK cells in vivo are less effective against subcutaneous growing tumors than against tumor metastases in visceral organs (22).

In the past, various antibodies have been used to deplete NK cells in vivo. The first antiserum to be used for in vivo treatment of mice and rats was anti-asialo

GM_1. This is a rabbit antiserum that recognizes a glycosphingolipid, ganglio-*N*-tetraosylceramide (23) present on mouse (23, 24), rat (25), and human (7) NK cells. However, asialo GM_1 is not a specific marker for NK cells, since it has been found to be expressed on interstitial and alveolar lung macrophages (26, 27), activated peritoneal macrophages (28, 29), alloimmune cytotoxic T lymphocytes (30), peripheral blood T cells (25), and subpopulations of cells in thymus, bone marrow, and lymph nodes (31, 32).

Treatment of mice or rats in vivo with anti-asialo GM_1 antisera can provide effective depletion of NK activity without significantly affecting the function of T and B lymphocytes (33, 34). However, this is highly dosage dependent and high levels of anti-asialo GM_1 can also inhibit T cell functions (15). Habu et al. (33) showed that in anti-asialo GM_1-treated nude mice the incidence of tumor take as well as tumor growth were enhanced when syngeneic (RLm-1), allogeneic (YAC-1), and human tumors were transplanted subcutaneously. Gorelik et al. (22) found that suppression of NK activity with anti-asialo GM_1 did not influence the growth of local tumors in C57BL/6 mice inoculated in the footpad with B16 melanoma or 3LL tumor cells, but accelerated the development of spontaneous pulmonary metastases. Also, the number of metastatic foci in the lungs of anti-asialo GM_1-treated C57BL/6 mice increased 10 times after intravenous inoculation with B16 melanoma. Similar results have been observed by several other investigators (21, 35–38).

In addition to anti-asialo GM_1, an NK-specific alloantiserum, anti-NK-1.1, can also be used to deplete mouse NK activity. Pollack and Hallenbeck (39) observed a reduced tumor clearance in lungs, liver, and spleen after treatment with anti-NK1.1. Finally, the mAb OX8 (anti-CD8), which recognizes cytotoxic/suppressor T cells, thymocytes, as well as NK cells in the rat, has been used in NK depletion studies (40, 41). However, the disadvantages of using this mAb for depletion studies are obvious, since OX8 cannot distinguish cytotoxic/suppressor T cells from NK cells, and therefore, it does not allow a selective depletion of NK cells. Moreover, OX8 does not provide complete depression of NK activity in vivo (40, 41).

The use of mAb 3.2.3 for in vivo NK depletion in rats is preferable to anti-asialo GM_1 or OX8 because of its strong and long-lasting effect and its high selectivity for NK cells. So far we have obtained complete NK depletion in various rat strains including Fischer 344, ACI, and Lewis (data not shown), suggesting that the NK depletion with mAb 3.2.3 is not strain dependent. We believe, therefore, that in vivo treatment of rats with mAb 3.2.3 will be useful for studies on the functional role of NK cells in vivo, as well as their growth and differentiation from $3.2.3^-$ precursors.

Summary

We recently described a mAb 3.2.3 (IgG1), that recognizes a 60-kD dimeric molecule expressed exclusively on fresh and rIL-2-activated NK cells and polymorphonuclear cells (16). mAb 3.2.3 enhances cytolytic activity of NK cells against selected FcR^+ tumor target cells by reverse antibody-dependent cellular cytotoxicity (ADCC), indicating that it recognizes an important triggering site on NK cells. The in vivo treatment of F344 rats with mAb 3.2.3 intraperitoneally completely and selectively eliminated NK/ADCC function in the spleen and peripheral blood for up to 10 d after treatment. Total numbers and percentages of T cells, monocytes, or PMN

were not decreased and T cell function, as determined by Con A stimulation, was not affected. The reduction in NK function was associated with a decrease in the numbers of LGL and the expression of other NK-related cell surface markers including CD2, CD8, and asialo GM_1. Depletion of NK cells with 3.2.3 markedly decreased the survival of F344 rats injected intravenously with MADB106 mammary adenocarcinoma cells, but did not affect the subcutaneous growth of MADB106 tumors. These results indicate that mAb 3.2.3 (in contrast to anti-asialo GM_1 and OX8, which are less selective markers) will be useful for studies on the functional role of NK cells in vivo as well as their in vivo differentiation and origin from $3.2.3^-$ precursors.

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