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High expression of NKR-P1 is not an absolute requirement for natural killer activity in BDIX rats

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Abstract NKR-P1 has been identified as a triggering structure selectively expressed on rat natural killer (NK) cells and adherent lymphokine-activated killer (A-LAK) cells. In vivo treatment with anti-NKR-P1 monoclonal antibody (mAb 3.2.3) was shown to induce complete inhibition of NK cytotoxicity and elimination of LAK cell precursors in Lewis and Fisher rat strains. We investigated the effects of mAb 3.2.3 in a colon tumor model in BDIX rats. Inoculation of animals with mAb 3.2.3 even at very high doses induced a strong but incomplete inhibition of NK cytotoxicity in nylon-wool-non-adherent spleen and peripheral blood cells. Generation of adherent A-LAK cells from their spleen precursors was also strongly but not fully inhibited. We also investigated the effect of treatment with mAb 3.2.3 on the tumorigenicity of the NK-sensitive REGb cell line. When subcutaneously inoculated in syngeneic animals, REGb cells induce tumors that first grow for 2 weeks, then spontaneously regress and disappear. In contrast with previous results using anti-asialoGM1, no significant difference in tumor growth was observed between rats treated with mAb 3.2.3 and control animals, even with a long-term treatment. In vitro, mAb 3.2.3 exhibited the same incomplete efficiency. Nylon-wool-non-adherent spleen cells treated with mAb 3.2.3 plus complement were completely free of 3.2.3^{bright} cells, but retained a substantial NK activity and generated LAK cells after culture with IL-2. After an overnight incubation in standard medium of 3.2.3-depleted spleen cells, 3.2.3^{bright} cells were partially recovered and the NK cytotoxic activity, as well as the generation of LAK cells, was significantly enhanced. These results suggest that a strong expression of NKR-P1 is not required for BDIX mononuclear cells to exhibit NK function and generate LAK cells under IL-2 activation.

Key words NK cell · NKR-P1 · Rat · Colon tumor · Tumor regression

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Introduction

Natural killer (NK) cells are responsible for rejection of tumors and inhibition of metastasis in numerous experimental models [9, 11, 17]. Nevertheless, uniquely specific markers for NK cells are lacking. A monoclonal antibody (mAb 3.2.3) has been described that selectively recognizes rat NK cells [4]. The NKR-P1 antigen recognized by this mAb has been identified as a triggering structure for NK activity [23], and it is now accepted as the most reliable marker of rat NK cells [31]. In vivo treatment of Fisher and Lewis rats with mAb 3.2.3 has been proved to eliminate NK cells selectively [28, 30].

In this paper, we investigated the in vivo effects of mAb 3.2.3 on NK cells in a model of regressive tumors of colonic origin in rats. REGb is a clone isolated from the DHD-K12 cancer cell line that has been established from a colonic tumor induced by dimethylhydrazine in a BDIX rat [15, 3]. REGb cells are tumorigenic in nude mice, but, when subcutaneously injected into BDIX rats, they produce tumors that first grow then regress and disappear within 6 weeks. REGb tumor regression is thought to involve NK cells since (1) REGb cells are more efficiently lysed in vitro by NK cells than another clone (PROb) of the DHD-K12 cell line that produces progressive tumors in vivo [20], and (2) in vivo inhibition of NK cytotoxicity by anti-asialoGM1 allowed REGb cells to produce larger, late-stage regressive,

or even progressive tumors [24]. Our results show that mAb 3.2.3 is unable to modify REGb tumor growth and is also unable to abrogate NK activity fully in vivo or in vitro.

Materials and methods

Animals

Animals used in this work were inbred male or female BDIX rats bred in our laboratory by brother-sister mating since 1971. The studies reported in this manuscript have been carried out in accordance with the national rules for care and use of laboratory animals (Décret n°87–848 du 19/10/87).

Tumor cell lines

REGb is a clone derived from the DHD-K12/REG cell line [3]. This line was isolated from the DHD-K12 cell line [15] that was established from a colonic tumor induced with dimethylhydrazine in a BDIX rat [16]. When subcutaneously injected into syngeneic rats (4–6 months old), REGb cells give rise to tumors that begin to regress after 2 weeks and completely disappear after 6 weeks, without metastasis. REGb cells were cultured in Ham's F10 medium (BioWhittaker, Walkersville, Md.) supplemented with 40 mg/l gentamicin (Gentalline, Unilabo, Levallois, France) and 10% fetal bovine serum (FBS; Anval, Betton, France) and the adherent cells were subcultured every 1–2 weeks after detachment by trypsin. REGb cells were stored in liquid nitrogen following the second passage after cloning and we used them within 3 months after thawing. These cells are not stained by mAb 3.2.3, as checked by flow cytometry. The Moloney-virus-induced mouse T cell lymphoma YAC-1 cells [26] and the murine mastocytoma P815 [7] were used as reference NK-sensitive and NK-resistant target cells respectively. These non-adherent cell lines were grown in RPMI-1640 medium (BioWhittaker, Walkersville, Md.) supplemented with 1% antibiotic/antimycotic solution (Gibco, Grand Island, N. Y.), 10 mM HEPES, 3 mM glutamine and 10% heat-inactivated FBS (complete medium), and subcultured three times per week. All cell lines were checked for contamination by bacteria, *Mycoplasma* or fungi by fluorescent staining of extracellular DNA using Hoechst 33258 [6].

Preparation of lymphoid cells

Peripheral blood was drawn either from anesthetized rats through a distal tail section throughout the experiments or by cardiac puncture at the end of the experiments. Peripheral blood mononuclear cells (PBMC) were isolated using gradient centrifugation over Lympholyte (Cedarlane, Hornby, Canada). Spleens were aseptically removed, mechanically crushed through a sterile steel mesh, and passed over nylon-wool columns to remove monocytes/macrophages and B cells. Single-cell suspensions were prepared in complete medium.

Generation of A-LAK cells

The generation of adherent lymphokine-activated killer (A-LAK) cells was performed according to Vujanovic et al. [29]. Briefly, nylon-wool-non-adherent mononuclear spleen cells were cultured at 37 °C in 5% CO_2 in T-75 flasks (Falcon, Becton-Dickinson, Moutain View, Calif.) at an optimal density of 2×10^6/ml in 20 ml LAK medium, which was RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 50 μM 2-mercaptoethanol (Bio-Rad, Richmond, Calif.), 0.1 mM non-essential amino acids (Gibco, Grand Island, N. Y.), 1.0 mM sodium pyruvate (Gibco), 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin (Sigma, St. Louis, Mo.). Just before cell incubation, 1000 units/ml recombinant human interleukin-2 (IL-2; kindly provided by Roussel-Uclaf, Romainville, France) was added to the LAK medium. After 48 h, the medium (conditioned medium) and the non-adherent cells were decanted and the adherent cells were washed three times with 15 ml prewarmed (37 °C) LAK medium. The adherent cells were fed with a 1:1 v/v mixture of fresh LAK medium with fresh IL-2 and conditioned medium filtred through a 0.45 μm filter. Cells were grown at 37 °C, 5% CO_2 for a total of 6 days. The adherent (A-LAK) cells were harvested after 5 min with 10 ml 0.025% EDTA, washed, counted and suspended at the chosen concentration in LAK medium.

Cytotoxicity assay

Cytotoxicity was measured in a ^{51}Cr-release assay with slight modifications of the previously described method [20]. To obtain optimal ^{51}Cr uptake, target cells were seeded at a known density before labeling (REGb: 2.5×10^6/75 cm^2, 48 h before; P815, YAC-1: 2.5×10^6/25 cm^2, 24 h before). They were labeled by adding 0.2 ml $Na_2{}^{51}CrO_4$ (1 mCi/ml, 400–1200 Ci/g; Du Pont de Nemours, Les Ulis, France) to 2.5×10^6 cells in 0.5 ml complete medium, followed by 1 h (P815, YAC-1) or 19 h (REGb) incubation at 37 °C. Plates were incubated at 37 °C in a humidified 5% CO_2 atmosphere for 4 h (P815, YAC-1) or 16 h (REGb). Cytotoxicity was calculated by the formula: cytotoxicity (%) = 100 × (experimental release – spontaneous release)/(maximum release – spontaneous release).

Antibodies

mAb 3.2.3 was produced as previously described [4]. Unconjugated mAb Sp3HL [anti-(sheep red blood cells)] (Serotec, Oxford, England) was used as the unrelated IgG1. For fluorescence staining, cells were treated with unconjugated mAb followed by phycoerythrin-labeled $(F(ab')_2$ fragments of rabbit antibody against mouse immunoglobulins (Serotec). In a second set of experiments (generation of LAK cells and in vitro depletions), to achieve better sensitivity and discrimination between $3.2.3^{bright}$ and $3.2.3^{dim}$ cells, cells were stained with biotinylated mAb 3.2.3 followed with phycoerythrin-labeled streptavidin. In these experiments, the control mouse IgG1 was mAb MOPC-21 (anti-*Escherichia coli*), purchased from Caltag (San Francisco, Calif.). The optimal dilution of each antibody was determined by preliminary dose/response titrations.

Flow cytometry

Cell-surface stainings were performed through standard procedures and cell suspensions were analyzed on a FACScan flow cytometer (Becton Dickinson, Mountain View, Calif.).

In vitro depletion with mAb 3.2.3

Nylon-wool-non-adherent mononuclear spleen cells were incubated for 2 h with saturating amounts of mAb 3.2.3 plus complement. Depletion with magnetic beads was also performed, using anti-(mouse IgG) M450 Dynabeads (Dynal, Oslo, Norway). In both depletion procedures, cell viability was better than 95%.

Treatment of rats

BDIX rats received an intraperitoneal (i. p.) injection of mAb 3.2.3 ascites or normal mouse serum (control animals) in 1 ml Hank's balanced salt solution (HBSS), once a day for 3 days. Experiments were performed with increasing doses of mAb. In the first experiment, 12 rats were treated with 3×1.75 mg IgG1/kg body weight; 2 rats were sacrificed on days 1, 5, 8, 12, 15 and 26 and their PBMC as well as spleen cells were pooled and tested for cytotoxicity. In the second experiment, 8 rats were treated with 3×10 mg IgG1/kg; 2 rats were sacrificed on days 1, 10, 16 and 29 and individually tested for the cytotoxicity of their spleen cells. In tumor challenge experiments, 10^6 REGb cells in 0.5 ml Ham's F10 medium were inoculated subcuta-

Table 1 Cytolytic activity of non-adherent spleen cells from control rats and rats treated with mAb 3.2.3 ascites. BDIX rats received i. p. injections of normal mouse serum (control) or mAb 3.2.3 ascites (3.2.3) once a day for 3 days. At the indicated times after the last 3.2.3 injection, 2 rats per group were killed and tested individually for the cytolytic activity of their spleen cells against YAC-1, P815 and REGb target cells. Results are expressed as percentage cytotoxicity (mean ± SD in 2 rats) at E/T = 100

Time after the last 3.2.3 injection (days)	Cytotoxicity (%) against:					
	P815		YAC-1		REGb	
	Control	3.2.3	Control	3.2.3	Control	3.2.3
1	0±0	0±0	50± 4	11±8	45±2	18±1
10	1±1	2±2	35± 6	9±8	50±9	15±1
16	4±6	0±0	38±18	7±8	34±7	7±1
29	6±1	3±1	38± 4	48±8	31±6	32±0

neously in the anterior thoracic wall of the rats 1 day after the last injection of mAb at a dose of 15 mg IgG1/kg. Tumor volume was estimated once a week by measuring with calipers two perpendicular diameters ($a < b$) of the tumor and using the formula $v = a^2 \times b/2$ [25]. The effects of an extended treatment schedule were tested in another group of rats. A group of 14 BDIX female rats weighing 100–120 g received a subcutaneous injection of 10^6 REGb cells in the anterior thoracic wall on day 1. Seven animals received seven i. p. injections of 0.25 ml mAb 3.2.3 in 1 ml HBSS on days –2, –1, 0, 9, 18, 29 and 40; 7 control animals received normal mouse IgG1 instead of mAb 3.2.3. PBMC were tested for NK cytotoxicity and 3.2.3 surface expression 2 days after each mAb injection.

Statistical analysis

Student's *t*-test was used to determine the statistical significance of the measured cytotoxicities, by comparing the mean ^{51}Cr release in the presence of effector cells to the mean spontaneous release of the same target cells, as well as the statistical significance of the effect of 3.2.3 treatment by comparing the ^{51}Cr releases induced by effector cells from treated and non-treated rats. A comparison of the size of REGb tumors in rats treated with mAb 3.2.3 and in control rats, at different times after tumor cell injection, was also performed using Student's *t*-test. A χ^2-test was used to analyze the effect of mAb 3.2.3 on the progressive or regressive pattern of tumors induced by REGb cells.

Results

Effect of in vivo treatment of BDIX rats with mAb 3.2.3 on NK activity of spleen cells and PBMC

An initial experiment was carried out to check the effect of in vivo treatment of BDIX rats with mAb 3.2.3 on the NK cytotoxicity of mononuclear cells. In 12 rats treated with 3 × 1.75 mg IgG1/kg, mAb 3.2.3 inhibited the cytotoxic activity of spleen and blood mononuclear cells against YAC-1 and REGb target cells. However, the amplitude and the kinetics of this inhibition differed from those observed in F344 [28] or Lewis rats [30]. Inhibition was never complete; it did not reach its maximum on day 1 but only on day 5 (about 80%), after which it continued; it was still significant on day 15 but had disappeared by day 26 (data not shown).

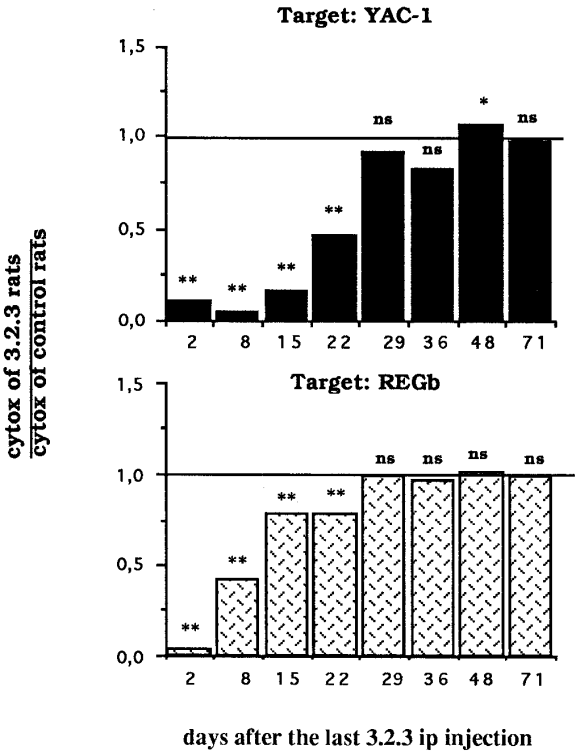

Fig. 1 Effect of a 3-day treatment of BDIX rats with mAb 3.2.3 ascites on the cytotoxicity (E/T = 100/1) of peripheral blood mononuclear cells against YAC-1 and REGb target cells. * $P < 0.02$; ** $P < 0.01$; *ns* not significant

Since a dose/effect relationship between the injected dose of mAb 3.2.3 and the inhibition of NK cytotoxicity was demonstrated in both F344 and Lewis rats, we performed a second experiment with a higher dose of mAb (3 × 10 mg IgG1/kg). Results depicted in Table 1 confirm the strong inhibitory effect of mAb 3.2.3 on NK activity in the spleen. This inhibition was of the same amplitude as in the former experiment but reached its maximum earlier: in YAC-1 cells (at E/T = 100) it was about 80% from day 1 to day 16, and had vanished by day 29. The cytotoxic effect of spleen cells on REGb target cells was similar in regard to their kinetics and amplitude. The cytotoxicity against P815 remained insignificant.

Effect of in vivo treatment with mAb 3.2.3 on subcutaneous REGb tumor growth

Ten animals were treated with 3 × 15 mg IgG1/kg before tumor cell inoculation. Every 7 days starting on day 2, blood was withdrawn by the tail section from four randomly chosen rats to test the cytotoxicity of their PBMC; on days 8, 15 and 71, one rat (chosen at random) was sacrificed and the cytotoxicity of its spleen cells was tested on the same target cells. A strong inhibition of the cytotoxicity of PBMC (Fig. 1) as well as spleen cells (Fig. 2) was observed in 3.2.3-treated animals. REGb tumor growth curves were similar in treated and control rats (Fig. 3).

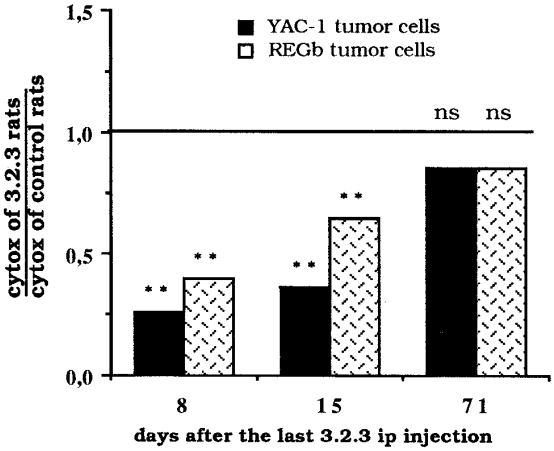

Fig. 2 Effect of a 3-day treatment of BDIX rats with mAb 3.2.3 ascites on the cytotoxicity (E/T = 100/1) of non-adherent spleen cells against YAC-1 and REGb target cells. * $P < 0.02$; ** $P < 0.01$; *ns* not significant

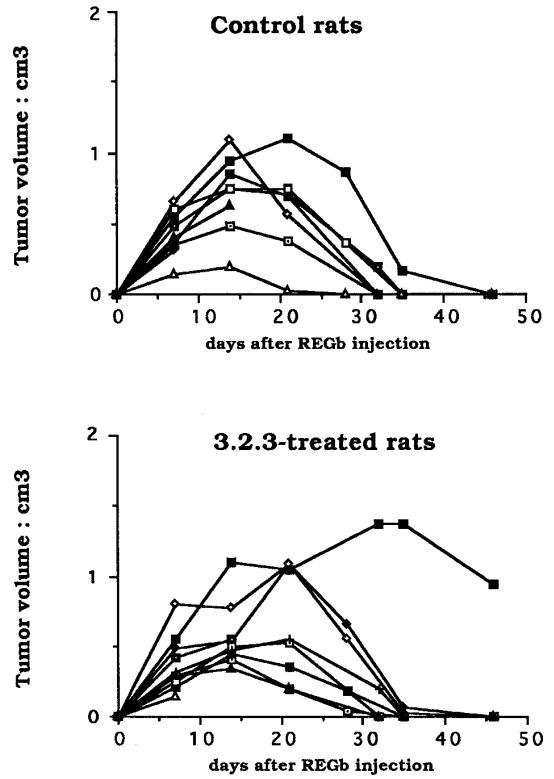

Fig. 3 Effect of a 3-day treatment of BDIX rats with mAb 3.2.3 ascites on the growth of REGb tumors. No significant difference (χ^2-test) was found between control and treated rats (10 rats per group)

Comparison of mean tumor volumes by Student's *t*-test showed no significant difference at any time from day 8 to day 35. χ^2 comparison of the numbers of progressive and regressive patterns in treated and control groups showed no significant difference.

Table 2 3.2.3-positive cells in spleen and blood from control and 3.2.3-treated rats challenged with REGb cells. Once a day for 3 days, BDIX rats received an i. p. injection of normal mouse serum (control) or mAb 3.2.3 ascites (3.2.3). On the next day, 10^6 REGb cells were s. c. inoculated in the anterior thoracic wall. At the indicated times after the last 3.2.3 injection, nylon-wool-non-adherent spleen cells and peripheral blood mononuclear cells (*PBMC*; purified by Lympholyte gradient) were stained with mAb 3.2.3 and analyzed by flow cytometry. Results are percentages of 3.2.3-positive cells

Time after the last 3.2.3 injection (days)	Positive cells (%)			
	PBMC		Spleen cells	
	Control	3.2.3	Control	3.2.3
2	24	6		
8	23	22	10	0
15	28	28	8	3
22	29	30		
29	23	21		
36	26	25		
48	17	17		
71	19	18	13	9

Treatment with mAb 3.2.3 induced a marked diminution of 3.2.3-positive cells in PBMC (Table 2): 6% versus 24% in control rats at day 2. This diminution did not last as long as the functional inhibition induced by 3.2.3, since a complete recovery was obtained as early as day 8. In the spleen, their kinetics seemed different since the number of 3.2.3-positive cells was still diminished by day 15; on day 71 the number of 3.2.3-positive cells was similar in treated and control animals.

Since the cytotoxicity of PBMC and spleen cells against YAC-1 and REGb cells was not fully inhibited on day 15 after the last injection of mAb 3.2.3, another in vivo experiment with repeated injections of mAb was performed in order to obtain a continuous inhibition of NK activity. This treatment strongly inhibited the cytotoxicity of PBMC against REGb and YAC-1 cells from day 2 to day 42, compared to control animals (Fig. 4). Despite this continuous inhibition, the growth curves were quite similar to those obtained in the first experiment, and REGb tumor growth did not significantly differ in treated and control rats (data not shown).

Effect of in vivo treatment with mAb 3.2.3 on A-LAK cells

In another experiment, four groups of 10 rats were treated according to the previous schedule: after i. p. injections of mAb 3.2.3 (groups A and C) or normal mouse IgG (groups B and D), animals were subcutaneously inoculated with REGb cells (groups A and B). On days 2, 9, 16 and 56, one rat randomly taken from each group was sacrificed, and spleen cells were tested for their NK cytotoxicity and their ability to generate A-LAK cells after IL-2 activation. The phenotype of A-LAK cells was then analyzed by flow cytometry and they were tested for cytotoxicity against

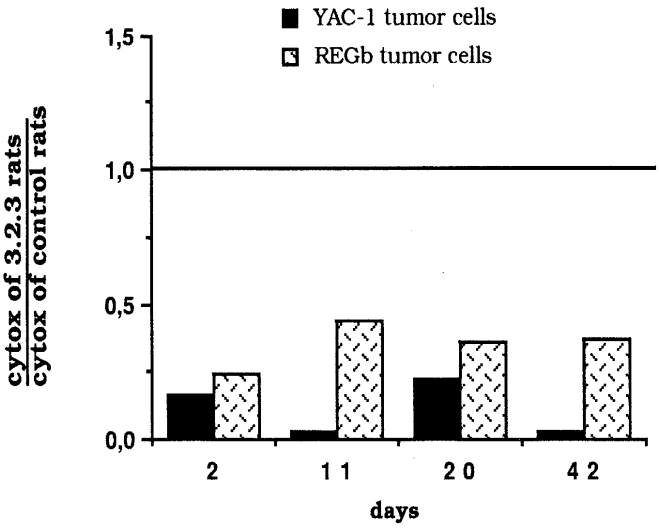

Fig. 4 Effect of a continuous treatment with mAb 3.2.3 ascites on the cytotoxicity of peripheral blood mononuclear cells (E/T = 100/1) against YAC-1 or REGb target cells. All inhibitions were significant ($P < 0.001$)

Table 3 Number of adherent lymphokine-activated killer (A-LAK) cells recovered after interleukin-2 (IL-2) activation of spleen cells. Effect of treatment of BDIX rats with 3.2.3 ascites on the number of A-LAK cells generated from spleen cells. Nylon-wool-non-adherent mononuclear spleen cells were cultured at an optimal density of 2×10^6 cells/ml in LAK medium, supplemented by 1000 U/ml recombinant IL-2. After 48 h, non-adherent cells were decanted, adherent cells were washed and fed with a 1:1 v/v mixture of fresh LAK medium with IL-2 and conditioned medium during an additional 4 days of culture. At this time, A-LAK cells were recovered and counted. Groups A and C were injected with 3.2.3, whereas groups B and D were treated with normal mouse serum. Groups A and B were also injected with REGb cells i. p. No difference was found between the REGb-injected and non-injected groups. Results are numbers of cells counted on four different days after the last 3.2.3 injection.

Group	$10^5 \times$ A-LAK cells			
	Day 2	Day 9	Day 16	Day 56
A	25	10	14	42
B	40	42	38	46
C	20	13	19	48
D	55	60	62	51

the reference LAK-sensitive P815 cells. No difference was found between REGb-injected and non-injected groups of rats. A strong inhibition of the NK cytotoxicity of spleen cells (data not shown) was observed in 3.2.3-treated animals, with the same kinetics and amplitude as in the previous experiments. The number of A-LAK cells recovered was reduced during the first 10 days, then increased and reached the same level as in control rats by day 56 (Table 3). The activity of A-LAK cells obtained from 3.2.3-treated animals was also strongly inhibited with the same kinetics (Fig. 5). The percentage of $3.2.3^{bright}$ cells among A-LAK cells recovered from 3.2.3-treated rats also followed the same kinetics (Fig. 6a), with only 15%–20% during the first 10 days, and 40% on day 16. On day 56 they reached the level (80%) observed in A-LAK cells obtained from control rats. Spleen cells from normal rats used as controls in each FACS analysis always contained 4%–6% $3.2.3.^{bright}$ cells (Fig. 6b).

In vitro kinetics of 3.2.3-positive cells from animals treated in vivo

The apparent inability of the in vivo treatment with high doses of mAb 3.2.3 to abolish NK activity completely could have been attributed to a re-expression of NKR-P1 by effector cells in vitro during the cytotoxicity assays. Such an artifact could also have explained why the cytotoxicity of spleen cells from treated animals was less inhibited when measured against REGb cells in a 16-h assay than when measured against YAC-1 cells in a 4-h assay. To rule out this possibility, we performed a kinetic study of the 3.2.3 phenotype of spleen and blood mononuclear spleen cells recovered 7 days after in vivo treatment. No significant amounts of $3.2.3^{bright}$ cells were found in either population during the first 24 h after spleen or blood collection and the $3.2.3^{dim}$ populations remained unchanged (Table 4).

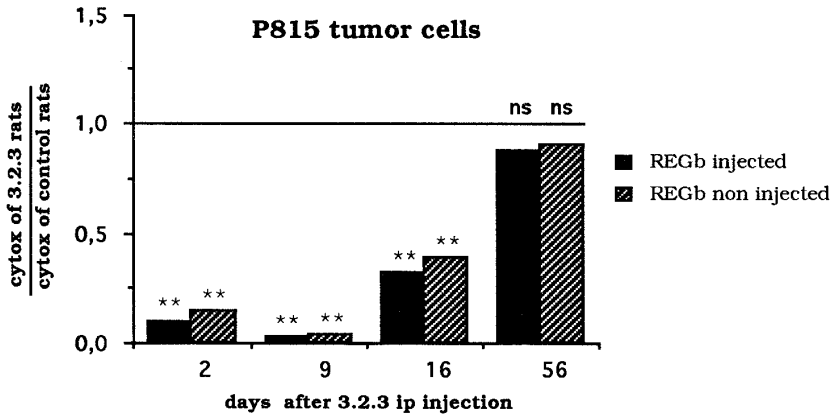

Fig. 5 Effect of treatment of BDIX rats with mAb 3.2.3 ascites on the cytotoxicity (E/T = 12.5/1) of adherent lymphokine-activated killer (A-LAK) cells against P815 target cells. No significant difference was found between rats inoculated with REGb cells and control rats. * $P < 0.02$; ** $P < 0.01$; *ns* not significant

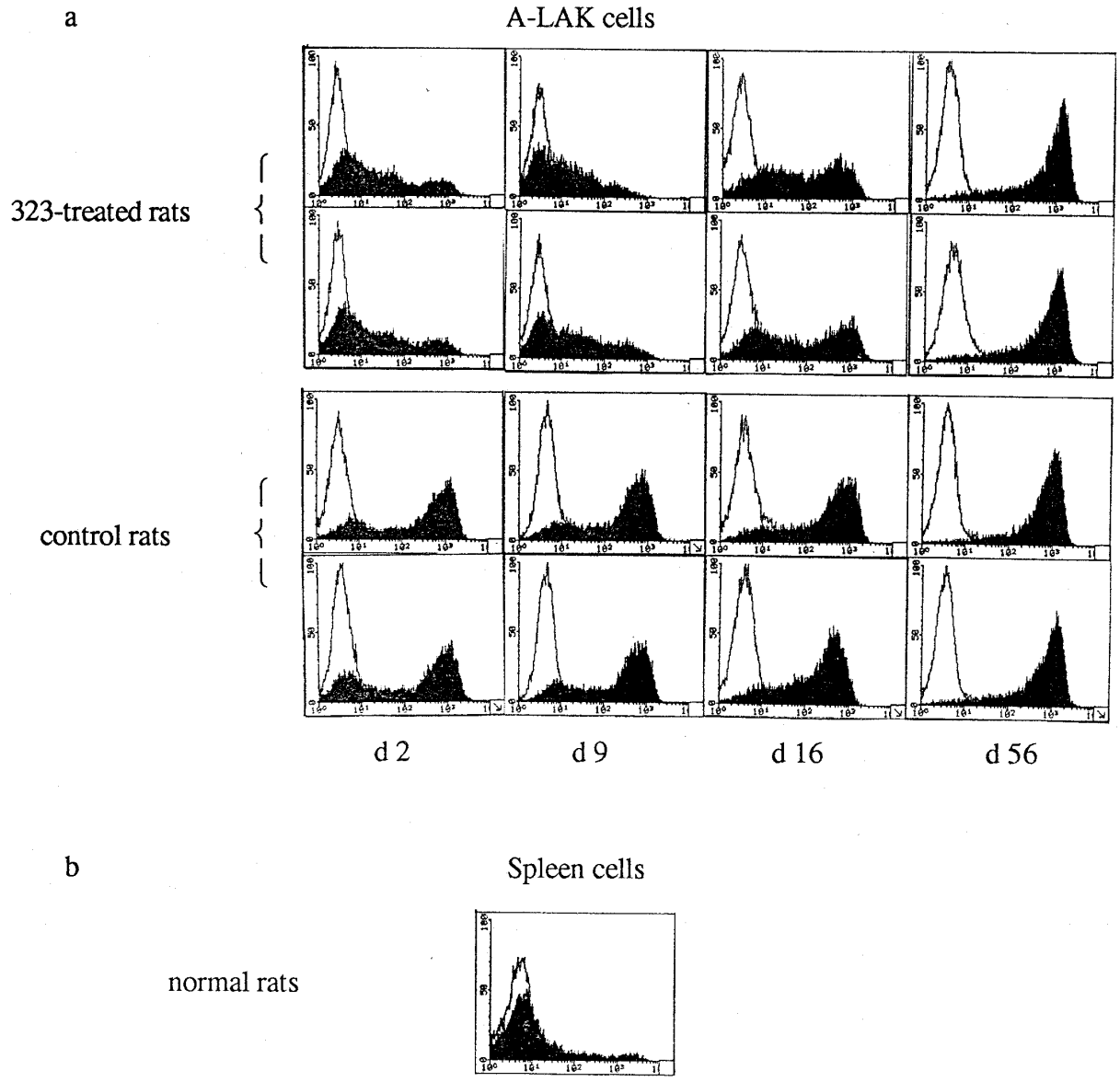

Fig. 6 a, b Effect of treatment of BDIX rats with mAb 3.2.3 ascites on the surface expression of NKR-P1 by A-LAK cells (**a**). Normal spleen cells are used as positive controls in each fluorescence-activated cell sorting analysis (**b**). Dark trace 3.2.3-labeled cells; *white trace* cells labeled with unrelated IgG1. For each group of rats (3.2.3-treated and control) the upper lane corresponds to REGb-injected animals

Effects of in vitro treatment of spleen cells with mAb 3.2.3 plus complement

The incomplete inhibition of NK cytotoxicity and LAK generation by in vivo treatment with mAb 3.2.3, prompted us to check whether an in vitro depletion of BDIX spleen cells allowed a complete elimination of $3.2.3^+$ cells and NK cytotoxicity. Total and depleted spleen cells were tested for their cytotoxicity and NKR-P1 expression immediately after depletion, after overnight incubation in standard complete medium (without IL-2), and after a 6-day culture with IL-2.

Immediately after depletion the cytotoxicity of depleted cells was significantly but incompletely inhibited (Fig. 7a). The $3.2.3^{bright}$ population was completely deleted, whereas only a minor proportion (about one-third) of the $3.2.3^{dim}$ cells was deleted. After overnight incubation in standard medium, 3.2.3-depleted cells recovered the same cytotoxicity as total non-depleted cells (Fig. 7b). Flow-cytometric analysis revealed the same percentage of $3.2.3^{bright}$ cells in depleted and in total cells, though their mean fluorescence intensity was weaker in depleted than in total cells (161 versus 208 arbitrary units). After activation with IL-2, 3.2.3-depleted cells gave rise to A-LAK cells with the same level of cytotoxicity against P815 cells, and the same 3.2.3 phenotype as total cells (data not shown).

Table 4 In vitro kinetics of 3.2.3-positive cells from a rat depleted of 3.2.3 in vivo. Spleen and PBMC were collected in a 3.2.3-treated female BDIX rat at day 7 after the last of three daily 3.2.3 i. p. injections. In vitro expression of NKR-P1 was checked with mAb 3.2.3 every 2 h for 24 h. Two populations were analyzed: cells with high NKR-P1 expression (3.2.3-bright) and cells with low expression of NKR-P1 (3.2.3-dim). Results are percentages of positive cells (parentheses show the mean of fluorescence of dim cells in arbitrary units)

Time (h)	PBMC		Splenocytes	
	3.2.3-bright (%)	3.2.3-dim (%)	3.2.3-bright (%)	3.2.3-dim (%)
3	0.1	17.0 (26)	0.0	7.8 (47)
5	0.0	15.6 (49)	0.2	10.3 (63)
7	0.1	16.0 (61)	0.2	8.0 (59)
9			0.6	9.2 (65)
11			0.6	9.4 (63)
13			0.6	9.7 (60)
15	0.3	16.1 (75)	0.6	9.7 (63)
17	0.2	15.1 (80)	0.6	9.3 (60)
19			0.2	9.6 (53)
24	0.1	12.5 (84	0.6	10.0 (60)
Control	5.2	18.0 (79)	4.1	10.1 (62)

Discussion

The monoclonal antibody 3.2.3 (anti-NKR-P1) obtained by Chambers et al. strongly reacts with 95% rat large granular lymphocytes and A-LAK cells (i. e. NKR-P1^{bright}). It also reacts with 80% polymorphonuclear leukocytes (PMN) but with a much weaker staining [4]. mAb 3.2.3 also recognizes a minor subset of T cells characterized as NKR-P1^{dim}, which are responsible for MHC-unrestricted cytotoxicity by T cells [2]. NKR-P1 has been identified as a disulfide-linked homodimer of 60 kDa [8], belonging to the same family of NK-related proteins as mouse A1 and human NKG2 [13, 5]. In vitro, F(ab′)$_2$ fragments of mAb 3.2.3 are unable to modulate NK cytotoxic activity [4]. On the other hand, in two rat models, in vivo treatment with mAb 3.2.3 completely eliminated NK function in the spleen and peripheral blood for up to 26 days [28, 30]. Furthermore, this treatment markedly decreased the survival of F344 rats injected intravenously with MADB106 mammary adenocarcinoma cells [28]; it also facilitated the growth of rat kidney carcinomas grafted in Lewis rats [30].

In our model, neither antibodies nor cytotoxic T lymphocytes specific for the REGb colon tumor cells could be demonstrated in tumor-bearing animals [21]. In contrast, several observations suggested that NK cells may play an important role in the control of REGb tumor

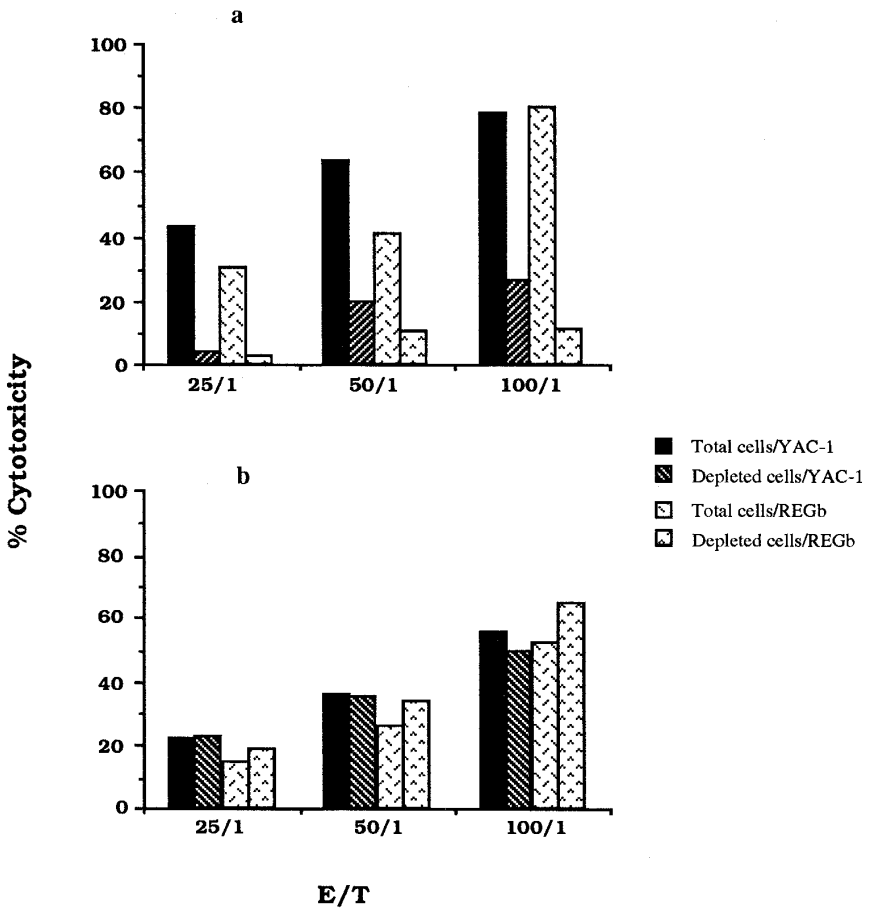

Fig. 7a, b Effect of in vitro treatment of BDIX mononuclear spleen cells with mAb 3.2.3 plus complement on their cytotoxicity to YAC-1 and REGb cells. Cytotoxicity was measured immediately after depletion (**a**) or after overnight incubation in standard medium (**b**). Cytotoxicity of depleted cells on both targets was significantly inhibited ($P < 0.001$) only when recovered immediately after depletion

regression. First, REGb cells are sensitive to BDIX NK cells in vitro whereas PROb cells, originating from the same cancer cell line and inducing progressive tumors in vivo, are fairly resistant [20, 19]. Second, treatment of rats with anti-asialoGM1 dramatically modified the REGb tumor growth, i. e. in 7 out of 10 rats a subcutaneous injection of REGb cells induced larger tumors, which regressed later, and in 3 out of 10 it even gave rise to progressive tumors [24]. However, no significant modification of NK cytotoxicity was observed in BDIX rats during REGb tumor regression. Furthermore, although anti-asialoGM1 reduces NK activity in several models in vivo [10, 14], it is also expressed by different non-NK cell types such as cytotoxic T cells [22, 27], monocytes/macrophages [22, 18, 1], and PMN [22]. This broad pattern of expression is correlated with the high percentage of asialo-GM1$^+$ cells in rat splenocytes when tested by fluorescence-activated cell sorting analysis: 74 $\pm$ 5% in F344 rats [28], which is similar to our findings in BDIX rats.

So mAb 3.2.3 appeared to be a most valuable tool to investigate more specifically the role of NK cells in the regression of REGb tumors. Rats were treated with mAb 3.2.3 in a protocol similar to that used with anti-asialoGM1. Since REGb tumors begin to regress at day 14, any effect of NK cells should take place very early after inoculation of REGb cells. Indeed, the cytotoxicity of PBMC against REGb cells was reduced for at least 15 days. This inhibition was stronger and longer-lasting in 3.2.3-treated animals, compared to that previously observed in anti-asialoGM1-treated rats: more than 95% versus 40% at day 2, 60% versus 40% at day 8, 25% versus 20% at day 15, and 25% versus 0% at day 22. The cytotoxicity of splenocytes against REGb cells was also strongly reduced up to day 15 in 3.2.3-treated rats, whereas no significant inhibition was obtained by day 10 in animals treated with anti-asialoGM1.

A strong (85%) inhibition of the cytotoxicity of PBMC against YAC-1 cells was also observed in BDIX rats during the first 2 weeks after a 3-day-treatment with mAb 3.2.3. However, unlike the results obtained in F344 and Lewis rats, this inhibition was never complete. Such a complete inhibition is unlikely to be obtained by the use of higher doses of mAb 3.2.3, since we obtained similar results with 1.75, 10 and 15 mg IgG1 (kg rat body weight)$^{-1}$ injection^{-1}. These huge doses are far greater than the highest one used in F344 rats (4 mg kg^{-1} injection^{-1}) [28]. In the same strain, an incomplete inhibition of NK cytotoxicity in spleen cells after two or three daily injections of 150–175 µl mAb 3.2.3 ascites was reported [12], but the IgG1 concentration and the number of treated animals were not precised. A complete inhibition of NK cytotoxic activity of Lewis rats was also obtained after a single injection of 25 µl mAb 3.2.3 ascites, the exact IgG1 concentration of which was not described [30]. In our experiments, even repeated injections of mAb 3.2.3 for 40 days were unable to induce a complete inhibition of NK activity in BDIX rats. This suggests that BDIX rats are less sensitive to the NK-inhibiting effects of mAb 3.2.3 than are Lewis or F344 strains, although such strain differences in the expression of NKR-P1 have not been documented in rats, since F344 is one of the most widely used strains and mAb 3.2.3 was generated by immunizing mice with F344 LAK cells [4]. In mice the NK1.1 antigen, which is highly related to the rat NKR-P1, is only expressed in a few strains [31].

As in F344 rats, mAb 3.2.3 also induced a marked diminution of 3.2.3-positive cells in the peripheral blood of BDIX-treated rats. The kinetics of this inhibition differed from that of the NK activity in PBMC, which was longer-lasting. This suggests that a re-expression of detectable levels of NKR-P1 by NK cells is not sufficient for a full functional recovery. The low number of 3.2.3$^+$ cells in the spleen at day 15 also suggests different kinetics of elimination compared to PBMC. The residual 3.2.3-positive cells in the blood and spleen of treated animals may consist of typical NK cells or of T lymphocytes expressing low numbers of NKR-P1 molecules [2, 28, 30].

Despite the strong effects of mAb 3.2.3 on NK cell number and function, and whatever the treatment schedule used, REGb tumor growth did not significantly differ in control and 3.2.3-treated rats. These results are in contrast with the enhancing effects of anti-asialoGM1 on the growth of REGb tumors. This discrepancy suggests that the control of REGb tumor growth requires a cooperation between NK and non-NK cells. Although the anti-asialoGM1 treatment induces a weaker suppression of NK activity, it may also suppress some non-NK cells (cytotoxic T lymphocytes, PMN?) on which the more specific mAb 3.2.3 may be less effective.

The relative in vivo inefficacy of mAb 3.2.3 prompted us to test the effects of in vitro treatment of BDIX spleen cells. Nylon-wool-non-adherent spleen cells depleted with mAb 3.2.3 plus complement were free of 3.2.3^{bright} cells, but were still able to lyse YAC-1 and REGb target cells and to generate LAK cells after IL-2 activation. Cells depleted in vitro were also highly cytotoxic for the natural cytotoxicity (NC)-sensitive WEHI-164 cell line (data not shown) suggesting that at least a part of the remaining cytotoxicity against REGb cells after 3.2.3 treatment may be due to NC-like cells. These results are again in contrast with those published for F344 rats showing that mononuclear 3.2.3^{dim} spleen cells do not exhibit NK cytotoxicity unless stimulated with IL-2 [2]. Any inadequacy of our depletion technique seems unlikely since similar results were obtained with twofold higher concentrations of mAb 3.2.3 and complement for the same cell number, as well as for mononuclear cells depleted with magnetic beads. Furthermore, after an overnight incubation in standard medium (without IL-2) 5%–8% 3.2.3^{bright} cells were found among the depleted cells, although their mean fluorescence intensity was lower than that of non-depleted cells. These "rested" cells also recovered a level of cytotoxicity against YAC-1 or REGb cells similar to that of non-depleted cells. In vivo treatment of animals with mAb 3.2.3 induced a more long-lasting inhibition of NKR-P1 expression since only insignificant numbers of 3.2.3^{bright} cells could be found in spleen or blood mononuclear cells 24 h after their collection. Altogether, these results strongly suggest that in the BDIX strain a substantial proportion of the NK

activity of blood or spleen mononuclear cells does not belong to the 3.2.3^{bright} population. As a correlate, strong expression of NKR-P1 may not be an absolute requirement for spleen or blood mononuclear cells to express NK cytotoxicity and generate LAK cells in IL-2 enriched medium.

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