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The influence of cyclophosphamide on antitumor immunity in mice bearing late-stage tumors

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Abstract. Spleen cells from mice bearing late-stage methylcholanthrene-induced tumor did not show any tumor activity when mixed with tumor cells in Winn's assay. Treatment of these mice with cyclophosphamide (CY) induced a tumor-inhibitory activity in spleen, occurring on day 7 after treatment, reaching its maximum on day 11 and disappearing by day 21. This antitumor activity could not be induced in control, tumor-free or T-deficient tumor-bearing mice. CY-induced tumor-inhibitory activity was immunologically specific, and mediated by Thy-1$^+$, L3T4$^-$, Ly-2$^+$ cells. Contrary to spleen cells from untreated tumor-bearing mice, spleen cells from CY-treated tumor-bearing mice did not suppress the antitumor activity of immune spleen cells in Winn's assay. However, in contrast to immune spleen cells, CY-induced tumor-inhibitory cells did not manifest antitumor activity when transferred systemically (i.v.) into T-cell-deficient tumor-bearing mice. Even more, spleen cells from CY-pretreated mice, harvested 7–15 days after the drug administration, partially suppressed the antitumor activity of concomitantly transferred spleen cells from specifically immune mice. Nevertheless, CY-pretreated mice manifested concomitant immunity, i.e. these mice exhibited higher resistance to a second inoculum of the same tumor than did nontreated mice or even mice with excised primary tumor.

Key words: Cyclophosphamide – Tumor immunity – Suppressor cells

Introduction

It is well known that animals bearing transplanted antigenic tumors develop specific antitumor immunity in the early stage of tumor growth, and that this immunity gradually disappears with further tumor growth. In mice transplanted s.c. with 1×10^6 cells, the antitumor activity of spleen cells from tumor-bearing mice is maximal on day 9 after tumor inoculation and disappears by day 14 [29]. The disappearance of tumor-specific immunity in mice with late-stage tumors, sometimes called "eclipse" [36], has been ascribed to various tumor-escape mechanisms [14, 36] and most recently to the action of suppressor cells.

Cyclophosphamide (CY) is one of the agents that is frequently used for modulation of various immunological disorders, including cancer [1, 16, 24, 25]. The immunomodulating effect of CY is most probably mediated by its preferential action on a host's suppressor cells [3, 28, 30, 31]. The enhancing effect of CY on antitumor immunity has been observed in a variety of host/tumor models [8, 9, 11, 15, 28, 38]. However, the ultimate effect of CY on antitumor immunity (i.e. enhancement or suppression) is greatly dependent on the time of its administration in relation to injection of tumor or immunizing inoculum. If given before tumor inoculation [13] or immunization to tumor [8, 11] CY generally increases the subsequent resistance of mice to tumor. On the other hand, if given after tumor (antigen), the effect of CY depends on the drug dose and the time of its application. Thus, if given in high sublethal doses (100–300 mg/kg) 1–2 days after tumor inoculation, CY can induce a state of specific tolerance to tumor [6, 35]. If given later at the same doses, i.e. to mice with established tumors, CY preferentially inhibits the action of suppressor cells, as shown in various tests in vitro [18, 38] and several models of tumor adoptive immunotherapy [2, 9, 28, 29]. However, whether CY increases the host's resistance to tumor simultaneously with the reduction of suppressor cell activity is less clearly shown. This prompted us to investigate the action of CY on antitumor immunity in mice bearing late-stage tumors. Data showing CY to induce temporary recovery of specific immunity in these animals are reported. However, this immunity is demonstrable only upon local transfer of immunity (Winn's assay), and not upon systemic (adoptive) transfer of immunity.

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Materials and methods

Mice. Inbred mice from our animal colony (CBA/H Zgr and CBAT6T6 Zgm) were used in all experiments. Mice of both sexes were used at 3–5 months of age. For obtaining T-cell-deficient recipients, mice were subjected to thymectomy, whole-body irradiation and reconstitution with syngeneic bone marrow cells (TIR mice). Thymectomy was performed at 4–6 weeks of age and irradiation (8.5 Gy X-rays, delivered by a Phillips therapeutic machine at a dose rate of 0.8 Gy/min) 21–30 days later. Samples containing $(5-7)\times 10^6$ syngeneic bone marrow cells, pretreated in vitro with anti-Thy-1.2 monoclonal antibodies (clone HO-13-4), were injected 2–4 h after irradiation.

Tumors. Two syngeneic methylcholanthrene-induced fibrosarcomas, designated as Mch-13 and Mch-52, were used in experiments. The tumors were induced by subcutaneously injecting adult CBA mice with 0.5 mg or 0.25 mg 20-methylcholanthrene (Fluka A. G., Buchs S. G., Switzerland). The tumors were maintained by s.c. passages in vivo, and tumors at the 18–29th passage (Mch-13) and the 3rd passage (Mch-52) were used in experiments. All tumors were initiated by injecting mice s.c. with $(0.5-1.0)\times 10^6$ tumor cells.

Immunization to tumor. This was carried out by a somewhat modified procedure described by North [28]. Briefly, mice were given s.c. 1×10^6 tumor cells mixed with 100 μg killed Corynebacterium parvum (Wellcome, London). This resulted in tumor growth for about 10–15 days, after which the tumor regressed in 80%–90% animals. Mice were challenged with 1×10^5 tumor cells 7–10 days after tumor regression, and resistant animals (mice that were tumor-free for the next 20–30 days) were used as donors of immune cells.

Spleen cell suspension. Spleens from immune or tumor-bearing mice were removed aseptically and teased through a stainless-steel wire screen in cold minimum essential medium (Difco). After one wash, cells were filtered through three or four layers of cotton gauze, to remove residual clumps. Occasionally a small quantity of deoxyribonuclease was added (2 μg/ml) to prevent cell clumping.

Depletion of T cell subpopulations from spleen by treatment with monoclonal antibodies. Spleen cells were treated with monoclonal antibodies against Thy-1.2 (clone HO-13-4), L3T4 (clone 191-2) and Lyt-2.1 (clone 169-4-2) antigens. These antibodies were a kind gift from Dr. S. Jonjić (School of Medicine, Rijeka, Croatia). Samples containing 25×10^6 cells were resuspended in 1 ml diluted antibodies (final concentrations, 2 μg/ml for anti-Thy-1.2 antibodies and 20 μg/ml for anti-L3T4 or anti-Lyt-2.1 antibodies) and incubated for 30 min at +4° C. After washing, diluted guinea-pig complement (Imunološki Zavod, Zagreb) was added to the sediment (1 ml 1:10 diluted complement/1×10^8 cells), and the cells were incubated for 45 min in a water bath at 37° C. The cells were centrifuged and resuspended in the desired volume of minimal essential medium. In this procedure, anti-Thy-1.2, anti-L3T4 anti Lyt-2.1 antibodies killed 23%–31%, 13%–20% and 9%–16% spleen cells, respectively, from mice bearing advanced tumors. In some experiment, cell suspensions were subjected to the second cycle of treatment with anti-L3T4 or anti-Lyt-2.1 antibodies, which increased cell killing by about 20%.

Winn's assay. The total numbers of effector and tumor cells were mixed at the desired ratio (300:1 or 100:1). The mixture was centrifuged and resuspended in a predetermined volume to contain the desired number and ratio of cells in 0.1 ml. Mice were given 0.1 ml mixture s.c. onto ventral abdominal skin. For the suppressor cell activity testing, spleen cells from nontreated tumor-bearing mice were mixed at a particular ratio with immune spleen or spleen cells from CY-pretreated tumor-bearing mice and tumor cells. All further procedures were the same as for the mixture of effector and target cells only.

Systemic transfer of immunity. T-cell-deficient recipients were given $(0.5-1.0)\times 10^6$ tumor cells subcutaneously and 3–4 days later $(70-80)\times 10^6$ spleen cells intravenously in 0.5 ml. When suppressor cell activity was tested, spleen cells from tumor-bearing mice were given i.v. to the same recipients 1 day after immune cells. The antitumor activity of transferred immune cells was assessed by recording the incidence and growth rate of tumors. The growth rate of tumors was determined by measuring two perpendicular tumor diameters with a vernier caliper. The percentage suppression was calculated by comparing tumor growth inhibition in mice given spleen cells alone with the growth inhibition in mice given both immune and tumor-bearing spleen cells.

Cyclophosphamide. Endoxan (Asta-Werke, Brackwede, FRG) was dissolved in sterile isotonic saline at a concentration of 20 mg/ml. Mice were administered i.p. 0.01 ml solution/body mass, giving a final dose of 200 mg/kg.

Statistics. Tumor diameters in particular treatment groups were compared by Student's t-test, tumor incidence and mortality by a χ^2 test, using Yates' correction of the test when indicated and survival times by Mann-Whitney U-test. Differences at $P<0.05$ were considered significant.

Results

CY induces tumor-inhibitory activity in the spleen of mice bearing late-stage tumors

Previous results [19] have shown that spleen cells of mice bearing early-stage transplanted methylcholanthrene-induced tumors (MCh-13) manifest tumor-specific immunity in Winn's assay. This immunity appears around day 5, is maximal on days 9–10, and disappears completely between days 15 and 21. In this study the influence of CY on antitumor immunity in mice bearing late-stage tumor, i.e. 21 or more days after tumor inoculation, was investigated. Preliminary experiments had shown CY to induce a tumor-inhibitory activity in these mice only if given at a dose of 150 mg/kg or higher (data not shown).

A total of 80 mice were injected s.c. with 1×10^6 MCh-13 cells. On day 22 (tumor diameter 11–15 mm), half of these mice were given CY (200 mg/kg i.p.) and the other half saline. Six mice in each treatment group were separated for measurement of tumor growth, while others were used as spleen cell donors for testing of antitumor immunity in Winn's assay. On days 3, 7, 11, 15, 21 and 28 after CY or saline injection, 4–6 mice were sacrificed in each treatment group and their spleens used as effector cells in Winn's assay. Spleen cell suspensions were prepared, mixed with tumor cells and injected s.c. into 8–10 normal recipients. The ratio of spleen to tumor cells (effector to target ratio) was 100:1 and 300:1; the dose of tumor cells was 5×10^4 or 1×10^5 cells. Tumor incidence was determined 30 days after injection of the cell mixture.

As seen in Fig. 1 A, spleen cells from mice given saline did not show any antitumor activity in the next 15–20 days at either effector:target (E:T) cell ratio. On the other hand, spleen cells from mice treated with CY manifested antitumor activity during the period 7–21 days after CY injection. It was maximal on day 11 and better expressed at a higher E:T ratio (300:1; 10 out of 10 mice without tumor at this ratio). Tumor growth in donors of spleen cells is shown in Fig. 1 B; as seen, CY delayed the tumor growth by about 12 days, after which the tumor resumed growth at a somewhat slower rate than in saline-treated controls.

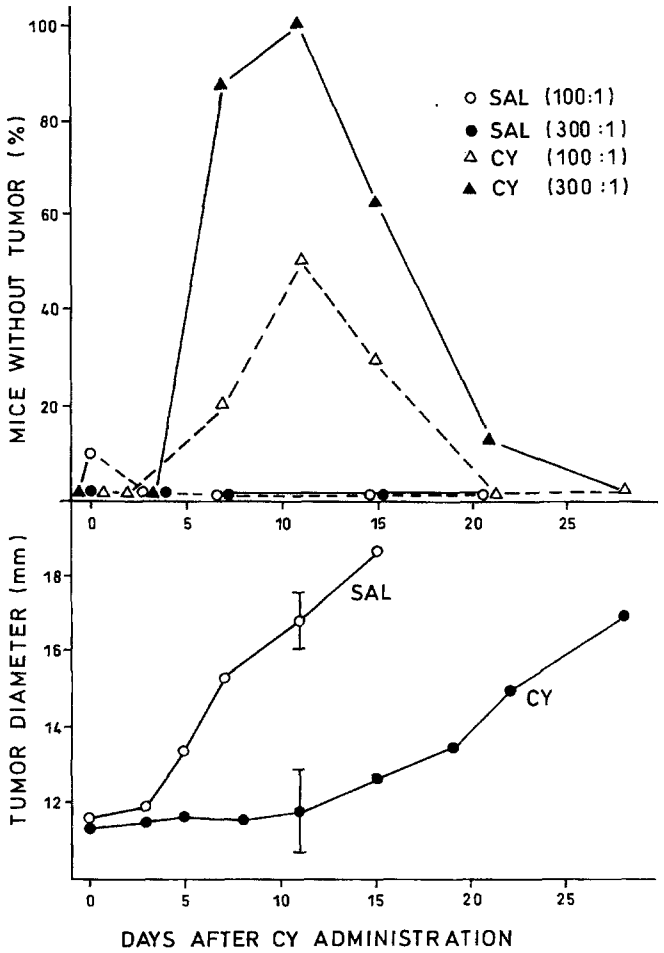

Fig. 1. A The antitumor activity of spleen cells from mice bearing late-stage tumors in Winn's assay. Mice were given either saline or cyclophosphamide (*CY*, 200 mg/kg i.p.) 22 days after inoculation of tumor cells (10^6 cell s.c.). **B** The growth of tumors in mice treated as in **A** ($n = 6$)

Table 1. Comparison of cyclophosphamide (CY) action on antitumor immunity of spleen cells from immunocompetent tumor-bearing mice, T-cell-deficient tumor-bearing mice and normal mice without tumor

Spleen cell donors[a]	Time between CY injection and spleen cell harvesting (days)	No. of mice without tumor/ total no. of mice[b]	Tumor diameter ± SE (mm)[b]
	–	0/8	13.5±1.4
Normal, without tumor	7	0/8	12.7±1.8
	11	0/8	11.3±1.1
Normal, with tumor	7	8/8	–
	11	8/8	–
TIR[c] with tumor	7	0/7	13.1±1.4
	11	0/8	10.2±1.1

[a] All mice were given 200 mg/kg CY
[b] Thirty days after injection of a mixture of spleen and tumor cells; ratio of spleen to tumor cells 300:1
[c] Mice thymectomized at 4–6 weeks, lethally irradiated and reconstituted with syngeneic bone marrow cells

Table 2. Specificity of antitumor immunity induced with CY

Tumor growing in spleen cell donors pretreated with CY[a]	Target tumor cells[b]	No. of mice with tumor, total no. of mice[c]
–	MCh-13	10/10
Mch-13	MCh-13	0/10
Mch-13	MCh-52	10/10
–	MCh-52	10/10
MCh-52	MCh-52	0/10
MCh-52	MCh-13	8/10

[a] Spleen cells harvested 11 days after treatment with CY (200 mg/kg)
[b] Ratio of effector to target cells 300:1
[c] Thirty days after s.c. injection of spleen/tumor cell mixture into normal recipients

CY-induced tumor-inhibitory activity is demonstrable only in immunocompetent tumor-bearing mice

In the next experiment, the effect of CY on spleen cell antitumor activity was compared in tumor-bearing normal or tumor-bearing T-cell-deficient mice and in control mice without tumor. Spleen cells were harvested 7 and 11 days after CY injection, and the E:T ratio was 300:1. Table 1 shows the incidence of tumors and mean tumor diameter on day 30. As seen, spleen cells from tumor-bearing normal mice prevented tumor growth in all mice, while spleen cells from tumor-bearing T-cell-deficient or control mice without tumor only slightly (but not significantly) inhibited the growth of tumors.

CY-induced tumor-inhibitory activity is immunologically specific

Table 2 shows the results of Winn's assay in which CY-treated donors of spleen cells were mice bearing either MCh-13 or MCh-52 tumors, and the tumor-inhibitory activity of their spleen cells was criss-cross tested against both tumors. It is clear that spleen cells from mice bearing MCh-13 tumors completely inhibited the growth of homologous MCh-13 tumor (10/10 recipients without tumor on day 30 after injection of the spleen/tumor cell mixture), but did not inhibit the growth of heterologous tumor (MCh-52) (10/10 recipients developed tumor; $P < 0.05$). A similar effect was obtained with spleen cells from mice bearing MCh-52 tumor; these cells inhibited the growth of MCh-52 tumor in 10/10 recipients, and the growth of MCh-13 tumors in 2/10 recipients ($P < 0.05$).

Tumor-inhibitory spleen cells induced with CY are Thy-1.2$^+$, L3T4$^-$ and Lyt-2.1$^+$ cells

Spleen cells from tumor-bearing mice pretreated 11 days earlier with CY were treated in vitro in the presence of complement with anti-Thy-1.2, L3T4 or anti-Lyt-2.1 monoclonal antibodies. Spleen cells were mixed with tumor cells (E:T ratio 300:1) and injected s.c. into normal recipients. Table 3 shows the incidence of tumors on day 32 after injection of the spleen/tumor cell mixture, obtained in two different experiments.

Table 3. Lyt-phenotype of CY-induced tumor-inhibitory cells

Treatment of spleen cells[a]	Incidence of tumors		
	Exp. 1	Exp. 2	Total
Complement (C′)	1/10[b]	2/10	19/20
Anti-Thy-1.2 + C′	10/10	10/10	20/20[c]
Anti-L3T4 + C′	1/10	3/10	4/20
Anti-Lyt-2 + C′	5/10	9/10	14/20[c]
No spleen cells added	5/ 5	5/ 5	10/10

[a] Spleen cells from tumor-bearing mice pretreated 11 days earlier with CY were exposed to antibodies and/or complement in one cycle (Expt. 1) or two cycles (Expt. 2), mixed with tumor cells (E:T ratio 300:1) and injected s.c. into normal recipients

[b] No. of mice with tumor/no. of mice without tumor 32 days after injection of spleen/tumor cell mixture

[c] Significantly different ($P < 0.01$) in relation to the group treated with complement alone

In the first experiment, spleen cells were exposed to antibodies and complement in one cycle. As seen in Table 3, the treatment with complement alone did not abrogate the tumor-inhibitory activity of spleen cells (tumor incidence: 1/10), while the treatment with anti-Thy-1.2 and complement abolished this activity completely (tumor incidence 10/10). Anti-L3T4 antibodies had no effect (tumor incidence 1/10), whereas anti-Lyt-2.1 antibodies abolished the tumor-inhibitory activity of spleen cells in 50% of recipients (tumor incidence 5/10). In the second experiment, spleen cells were exposed to the action of anti-T cell antibodies and complement in two cycles (see Materials and methods). As seen (Table 3), this treatment enhanced the activity of both anti-L3T4 and anti-Lyt-2.1 antibodies, but preferentially of the latter. Thus, tumor incidence was 3/10 and 9/10 in mice treated with anti-L3T4 and anti-Lyt-2.1 antibodies respectively. The pooled data from both experiments (Table 3) show that the treatment with anti-Thy-1.2 and anti-Lyt-2.1 antibodies significantly decreased the antitumor activity of spleen cells ($P < 0.01$), whereas the treatment with anti-L3T4 antibodies did not affect this activity significantly ($P > 0.05$).

Further experiments have shown tumor-inhibitory T cells from CY-treated tumor-bearing mice to be non-adherent to plastics, and they mostly separate into high-density fractions (above 1.083 g/cm^3) on Ficoll/Hypaque density gradients (data not shown).

CY-induced tumor-inhibitory activity in spleen of mice bearing late-stage tumors is not demonstrable upon systemic (i.v.) transfer of spleen cells

Four groups of T-cell-deficient (TIR) mice were given 1×10^6 MCh-13 cells s.c. After 4 days, three groups of mice were given i.v. 80×10^6 spleen cells from specifically immunized mice or mice bearing late-stage MCh-13 tumors pretreated 11 days earlier with either CY or saline. The fourth (control) group of mice was not treated further. Figure 2 shows that immune cells significantly inhibited the growth of tumor in TIR mice. On the other hand, tumor-bearing spleen cells pretreated with CY or saline

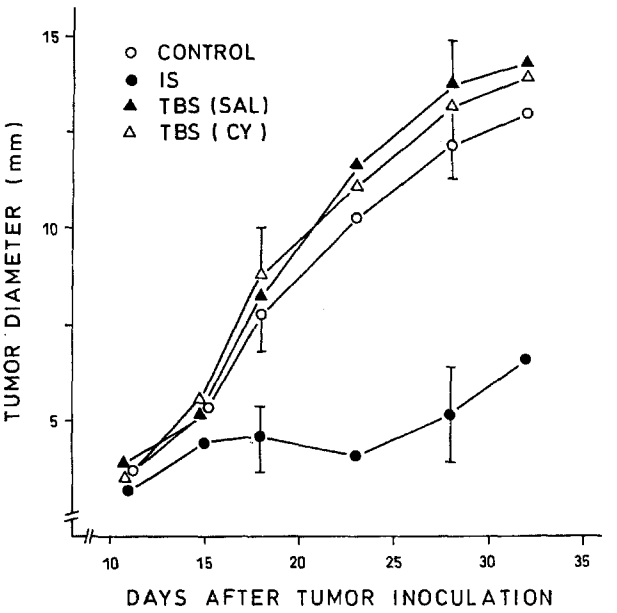

Fig. 2. The growth of tumors in T-cell-deficient mice given on day 3 i.v. 80×10^6 spleen cells from specifically immune mice (*IS*), or spleen cells from tumor-bearing mice pretreated 11 days before cell transfer with either saline [*TBS (SAL)*] or CY [*TBS (CY)*]. *Vertical bars* denote SE of the mean ($n = 5-6$ mice/group)

had no significant effect on tumor growth. This experiment was repeated twice with similar results, i.e., tumor growth in recipients receiving spleen cells (in one experiment up to 200×10^6 cells) from mice pretreated 11 days earlier with CY was similar or even faster than in untreated controls. On the other hand, immune spleen cells inhibited tumor growth significantly at a dose of 40×10^6 cells (data not shown).

The influence of CY on suppressor cell activity in spleen of mice bearing late-stage tumors

Mixing experiments were used for the testing of suppressor activity in Winn's assay, as well as upon systemic (i.v.) transfer of spleen cells. In Winn's assay, spleen cells from specifically immunized mice (immune spleen cells) were mixed at a constant ratio (50:1) with tumor cells. To these cells, spleen cells from mice bearing late-stage tumors pretreated 7 days earlier with either CY or saline were added, and the cell mixture was injected s.c. into normal recipients. Two ratios of tumor-bearing (suppressor) to immune spleen cells were used: 1:1 (i.e. ratio to tumor cells 50:50:1) and 3:1 (ratio to tumor cells 150:50:1).

Table 4 shows the time of tumor appearance and tumor incidence at day 60 after inoculation with the spleen/tumor cell mixture. Immune spleen cells alone inhibited tumor growth in all recipients (group 2). Spleen cells from tumor-bearing mice treated with saline suppressed the antitumor activity of immune spleen cells, but only when their ratio to immune cells was 3:1 (group 5). This suppression was not complete, since tumors developed in 6 out of 8 animals only and appeared later than in nontreated controls (group 1). However, spleen cells from CY-pretreated mice

Table 4. Influence of CY on the suppressor activity of spleen cells in Winn's assay

Group	Type and ratio of cells added[a]	Tumor growth	
		Incidence[b]	Time of appearance (days)
1	Tumor	0/8	8.0 (6–11)
2	IS+T (50:1)	0/8	–
3	IS+TBS(Sal)+ T(50:50:1)	0/8	–
4	IS+TBS(CY)+ T(50:50:1)	0/8	–
5	IS+TBS(Sal)+ T(50:150:1)	6/8	15.0 (14–22)[c]
6	IS+TBS(CY)+ T(50:150:1)	0/8	–
7	TBS(Sal)+ T(150:1)	8/8	7.5 (6– 9)
8	TBS(CY)+ T(150:1)	0/8	–
9	TBS(CY)+TBS(Sal)+ T(150:150:1)	0/8	–
10	TBS(CY)+TBS+ T(150:450:1)	5/8	19.0 (17–27)[c]

[a] IS, immune spleen cells; TBS(CY) and TBS(Sal), cells from tumor-bearing mice pretreated 7 days earlier with CY or saline respectively; T, tumor cells (1×10^5)

[b] No. of mice with tumor/total no. of mice, 60 days after tumor inoculation

[c] Significantly longer than in group 1 ($P < 0.01$)

Table 5. Concomitant immunity in tumor-bearing mice treated with CY

Primary tumor	Treatment[a]	Incidence of secondary tumor at a challenge dose		
		2×10^4	1×10^5	Total
+	Saline	5/ 8	0/ 9	13/17
+	CY	1/10	3/10	4/20[c]
+	Excision	3/ 5	7/ 9	10/14
–	–	9/10	10/10	19/20

[a] Fifteen days after inoculation of primary tumor (1×10^6 MCh-13 cells; tumor diameter 8–10 mm), mice were given 0.2 ml saline, 200 mg/kg CY or their tumors were surgically removed

[b] Secondary tumor was injected 6 days after the treatment

[c] Significantly different from group of mice given only secondary tumor ($P < 0.05$)

did not show any suppressor activity at any ratio to immune cells (groups 4 and 6). In further experiments, spleen cells from saline-pretreated tumor-bearing mice were tested for their suppressor activity on spleen cells from CY-pretreated tumor-bearing mice. Similarly to their effect on immune spleen cells, spleen cells from saline-pretreated mice suppressed the antitumor activity of spleen cells from CY-pretreated mice at a higher cell ratio only, and the suppression was not complete (groups 9 and 10, Table 5).

Similar results (i.e., no suppressor activity detected) were obtained when tumor-bearing spleen cells were used 1 or 11 days after CY administration (data not shown).

In the systemic transfer of immunity, TIR mice were used as recipients of immune and potential suppressor cells. On the 3rd day after tumor inoculation (1×10^6 Mch-13 cells s.c.), three groups of mice received 70×10^6 immune spleen cells. One day later, two groups of these mice received the same number of spleen cells from tumor-bearing mice treated 11 days earlier either with saline or CY, while a third group was not treated further (positive control). A fourth group of mice received only tumor cells (tumor-growth control). As in experiments shown in Fig. 2, immune spleen cells alone significantly inhibited tumor growth (Fig. 3). Spleen cells from tumor-bearing mice given saline abrogated entirely the antitumor activity of spleen cells. Surprisingly, the spleen cells from CY-pre-

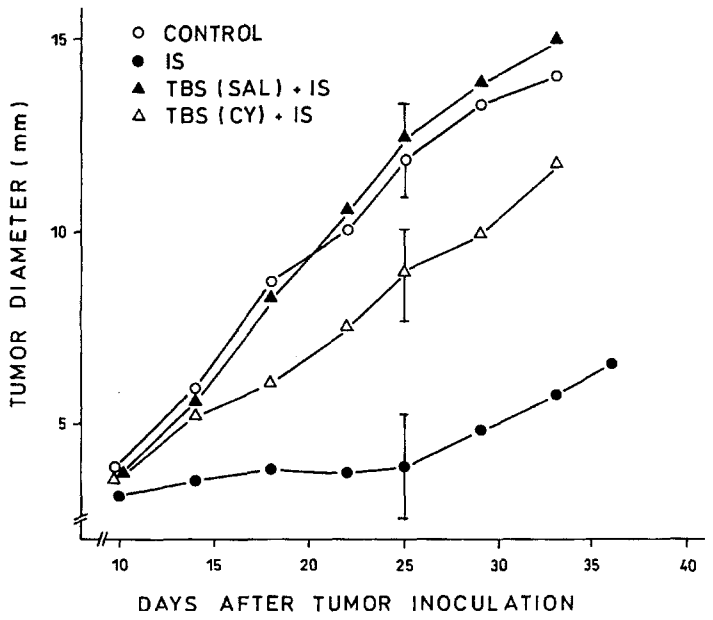

Fig. 3. Suppressor activity of spleen cells from CY-pretreated tumor-bearing mice in the model of systemic transfer of immunity. On the 3rd day after tumor inoculation (1×10^6 Mch-13 cells s.c.), thymectomized, lethally irradiated, bone-marrow reconstituted mice were given 70×10^6 immune spleen cells (*IS*) i.v. and 1 day later the same number of spleen cells from tumor-bearing mice, pretreated 11 days earlier with either saline [*TBS (SAL)*] or CY [*TBS (CY)*] ($n = 5–6$ mice)

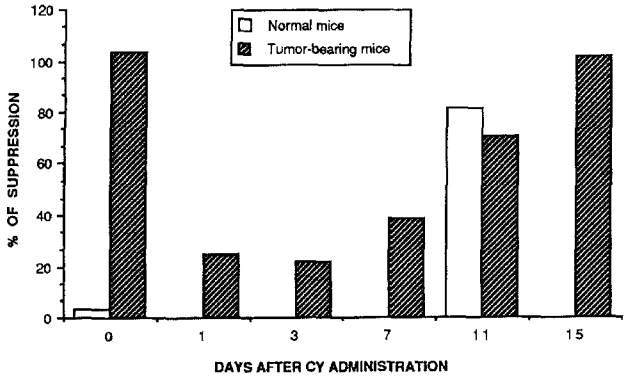

Fig. 4. Kinetics of suppressor cell activity in spleen of CY-pretreated tumor-bearing mice upon their systemic transfer. Experimental design was the same as in the experiment shown in Fig. 3

treated tumor-bearing mice also inhibited about 70% of the antitumor activity of immune spleen cells.

In the next experiment we investigated the kinetics of systemic suppressor cell activity after CY administration in tumor-bearing mice. As seen in Fig. 4, within 24 h CY eliminated most of the suppressor activity in the spleens of tumor-bearing mice. However, suppressor activity started to recover from day 7, reaching the pretreatment level on day 15. Interestingly, CY induced suppressor activity in spleen cells of normal mice, which on day 11 was somewhat higher than in spleen cells of tumor-bearing mice.

The influence of CY on concomitant immunity in mice bearing tumors

Mice were given 1×10^6 MCh-13 cells s.c. in the right flank; 15–17 days later (tumor diameter 8–9 mm), 6 mice were separated for measuring the growth of primary tumors, and the rest were treated as follows: one group was given saline, the other CY (200 mg/kg) and in the third the tumor was surgically removed. Six days after this treatment mice were given 2×10^4 or 1×10^5 MCh-13 cells s.c. in the left flank. The same number of cells were injected into two groups of normal mice (controls of the growth of secondary tumor). The growth of tumor was measured twice weekly and the incidence of secondary tumors was recorded on day 15 and, in animals that survived, on day 30.

As seen in Table 5, a high resistance to secondary tumor was observed in the group of mice bearing primary tumors, treated with CY. When the results obtained with two doses of secondary tumors were pooled, the incidence of tumors was significantly lower than in the control group (incidence 4/20 vs 19/20 respectively; $P < 0.05$). Surgical excision did not significantly decrease the incidence of secondary tumors (incidence 10/14 vs 19/20 in the control group or 13/17 in tumor-bearing mice treated with saline; $P > 0.05$). In all groups of mice bearing primary tumors, the growth of secondary tumors was slower than in the control group (data not shown).

Discussion

In the experiments, presented the effect of CY on antitumor immunity in mice bearing late-stage tumors was investigated. At the time of CY application, the animals did not manifest concomitant immunity to tumor, nor could their spleen cells inhibit tumor growth upon transfer locally (Winn's assay) or systemically (i.v.) into T-cell-deficient recipients. The tumor used in the experiments was moderately sensitive to the direct cytostatic effect of CY (Fig. 1 B). Administration of CY to these animals induced the generation of tumor-inhibitory cells, which inhibited the growth of autologous tumor in Winn's assay. The antitumor activity appeared on day 7 after CY administration, was maximal on day 11 and disappeared by day 21 (Fig. 1 A). These CY-induced tumor-inhibitory cells could only be induced in normal (immunocompetent) tumor-bearing mice and not in tumor-bearing T-cell-deficient mice or in normal, tumor-free mice (Table 1), indicating that CY-induced tumor-inhibitory cells are most probably T lymphocytes. This was more directly confirmed in experiments with depletion of T cell subsets. The tumor-inhibitory activity could be consistently abolished by treatment with anti-Thy-1 and anti-Ly-2 antibodies, but not with anti-L3T4 antibodies, indicating that they are Thy-1$^+$, Ly-2$^+$, L3T4$^-$ T lymphocytes (Table 3). The antitumor activity of CY-induced tumor-inhibitory cells was shown to be immunologically specific, since they inhibited the growth of homologous but not of heterologous tumor (Table 2). These cells were shown to be high-density cells that did not adhere to plastics (data not shown).

Although spleen cells from CY-pretreated tumor-bearing mice inhibited tumor growth in a model of local passive transfer of immunity (i.e. in Winn's assay), they had no effect in a model of systemic transfer of immunity (i.e. in the model of adoptive immunotherapy of tumors in T-cell-deficient recipients). This contrasted with spleen cells of specifically immunized mice, which were efficient in both models (Table 4 and Fig. 2) and [27]). The reason why CY-induced antitumor effector cells are acitve in local and inactive in systemic transfer assay is not clear. However, one can envisage several possible mechanism causing this dichotomy.

First, spleen cells from CY-pretreated tumor bearers, contrary to spleen cells from immune mice, may not have enough effector cells to be effective upon systemic transfer. Indeed, in Winn's assay, immune spleen cells are, on a cell-number basis, about six times more active than spleen cells from CY-pretreated tumor-bearing mice (unpublished data). However, this is probably not the main reason for the nonefficacy of CY-pretreated cells, since these cells had no tumor-inhibitory effect even when given i.v. in doses of up to 200×10^6 cells, whereas immune spleen cells were inhibitory at a dose of 40×10^6 cells. Second, the effectors in local and systemic transfer of immunity are distinct and CY affects these two type of effectors differently. In most [10, 12, 22], but not all [21, 29], experimental models the effector cells in systemic transfer of immunity were shown to be L3T4$^+$ or CD4$^+$ T lymphocytes, and Ly-2$^+$ or CD8$^+$ cells in local transfer of immunity. Literature data on the recovery of of various T cell subpopulations after high

doses of CY are scarce, but some point to delayed recovery of helper/L3T4+ function and rather quick recovery of cytotoxic/Ly-2+ function [4, 33, 35]. Our data have shown that effector cells in a local (Winn's) assay are Ly-2+ T lymphocytes. Third, CY perturbs the action of suppressor cells, which differently affects the effector cells in local and systemic transfer of immunity. Before CY administration, spleens of mice bearing late-stage tumors contained suppressor cells that efficiently inhibited the antitumor action of immune spleen cells upon their systemic transfer (Fig. 3), but were less active when transferred locally with immune spleen cells (Table 4). In other experiments, in the same [27] or a different [28] host/tumor model, i. v. transferred suppressor cells completely inhibited the action of transferred immune spleen cells even when their ratio to immune cells was 1:3. CY eliminated most of this suppressor activity within 24 h after its administration. However, from day 7 on, the recovery of suppressor activity in the spleen could be demonstrated in systemic but not in local transfer assay (Fig. 4 and Table 4). The fact that the same activity appeared in CY-pretreated normal mice (Fig. 4) indicates that it is CY-induced and mediated by natural suppressor cells. Others have shown that natural suppressor cells appear 5–7 days after CY administration and are maximally active during the next 1 or 2 weeks [23]. The inability of these cells to suppress the action of immune spleen cells in Winn's assay points again at different effectors and/or mechanisms operating in local and systemic transfer assay.

There are many data showing that spleen cells from tumor-bearing mice can inhibit the action of immune lymphoid cells upon their systemic transfer into immunosuppressed recipients, as well as that CY eliminates this suppressor activity within 24 h. However, data on the ability of the same cells to suppress the activity of immune cells in local transfer of immunity are controversial; in some models they they suppressed immune cells at the ratio of 1:1 [5], in others only at higher ratios [7] and in still others they had no suppressor effect [34]. Our data thus showed that the lack of apparent antitumor reactivity in mice bearing late-stage tumors [36] is not caused by loss of tumor-effector mechanisms [17] in these animals (at least at the level of local immunity), but most probably by its down-regulation by suppressor mechanisms.

In spite of different activities of spleen cells from CY-pretreated mice upon their local and systemic transfer, CY increased antitumor resistance at the level of the whole animal. Tumor-bearing mice pretreated with CY showed greater resistance to a second implant of the same tumor than non-treated mice or even mice with surgically removed tumor. These result are very similar to those reported by Steele and Pierce [32], who obtained essentially identical results in mice bearing early-stage tumors. They are also reminiscent of the data obtained in experiments with high dose tolerance to contact allergens [30, 31]. In these experiments high doses of CY (300 mg/kg) abolished the established tolerance to an antigen, but it reappeared 2–3 weeks after the drug administration. At present, it is difficult to explain, at the level of the whole animal, the interplay of various antitumor effectors detected in local and systemic transfer assays. Obviously, the complex perturbations of suppressor and other cells in lymphoid tissue of tumor-bearing mice after CY treatment should be studied in more detail.

The importance of recovery of antitumor after CY treatment might account for the better antitumor effect of CY in normal, immunocompetent mice than in mice with suppressed immune reactivity [20, 26].

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