Eur J Cancer Clin Oncol, Vol. 18, No. 4, pp. 405–411, 1982
Printed in Great Britain.

0277-5379/82/040405-07$03.00/0

Suppressor T Cells and the Regression Phase of Syngeneic Intradermally Developing P-815 Mastocytomas*

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Abstract—*Thymus cells obtained 6, 8 or 10 days after the intradermal injection of P-815 tumor cells into syngeneic DBA/2 mice were, upon adoptive transfer into new hosts, incapable of abrogating the normally observed tumor regression phase. Transfer of cells from day 8 revealed a tendency toward supression of regression phases; cells from day 6 or 10 were inert or had a rather immunopotentiating effect. Pretreatment of tumor hosts with cyclophosphamide did not result in more pronounced regression phases, nor did this treatment raise the percentage of surviving animals. Injection of cyclophosphamide after tumor cell inoculation resulted in a slight delay of tumor growth, but not in more pronounced regression nor in higher survival rates. Taken together the data suggest that in a weakly immunogenic and highly malignant tumor, supressor T cells may play a minor role in the subversion of the host's immune response.*

INTRODUCTION

THE INTRADERMAL development of P-815 tumors in syngeneic DBA/2 mice is characterized by a period of tumor growth followed by tumor regression. In some instances regression may be complete, but in most animals it is prematurely terminated and progressive growth eventually kills the tumor hosts. The cause of this regression has been assigned to the activity of killer T lymphocytes which develop in parallel with tumor growth and reach a maximum activity around days 10–12, i.e. when tumor regression begins. Why tumor regression is terminated and T cell cytotoxicity *in vitro* disappears, as does concomitant immunity, is still an unanswered question [1].

Growth of malignant cells, despite the induction of an immune reaction, is a general problem in tumor immunology. Besides subversive effects directly exerted by tumor cells, mechanisms involving macrophages, B cells and especially suppressor T cells have been postulated (for review see [2]). For the P-815 tumor model the stimulation of suppressor T lymphocytes has been demonstrated [3,4]. It was shown that spleen cells from late tumor-bearing animals and thymus cells from early tumor bearers of day 8 inhibit the secondary stimulation *in vitro* of killer T cells. However, the crucial question concerning the role which supressor T cells play in the regulation of the immune response in the tumor-bearing host is not answered by these studies. We therefore tested if adoptively transferred thymus cells from P-815 tumor-bearing animals were able to influence the development of i.d. injected tumor cells, especially if by such a manipulation the regression phases were abrogated and the survival times shortened.

Secondly, we attempted to improve the immune response of tumor-bearing animals by treating them with a single dose of Cy. Pretreatment with this agent has been demonstrated to result in the induction of cytotoxic T cells against TNP-modified syngeneic splenocytes *in vivo* [5]. Treatment after tumor cell injection has been shown to result in an otherwise not observable phase of regression in the syngeneic MOPC-315 tumor model [6]. The s.c. development of P-815 tumors could be modulated by low dose X-irradiation after tumor cell inoculation [7]. This effect was sup-

Accepted 21 December 1981.
*This work was supported by the Swiss National Science Foundation, grant number 3.486.79.
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Abbreviations used in this paper: cy, cyclophosphamide; DMEM, Dulbecco's modification of the Minimum Essential Medium; HBSS, Hanks' balanced salt solution; TNP, trinitrophenyl–; i.d., intradermal; i.p., intraperitoneal; i.v., intravenous; s.c., subcutaneous.

posedly achieved by affecting the radiosensitive population of suppressor T cells. Treatment with Cy was therefore carried out before and after tumor cell injection.

We found that transferred thymus cells from tumor bearers were not capable of suppressing the tumor regression phases, although they slightly enhanced tumor growth. On the other hand, Cy pretreatment did not improve tumor regression but resulted in larger tumors and shorter survival times. Cy treatment after tumor cell injection had a weak beneficial effect on the tumor size but not on survival rates.

MATERIALS AND METHODS

Mice

Female DBA/2 mice were purchased from Gl. Bomholtgard Ltd., Ry, Denmark, and used at the age of 12–16 weeks.

Tumor

The P-815X2 cells were banked in liquid nitrogen and cultivated in DMEM containing 10% horse serum.

Tumor cell injections

They were performed essentially as described previously [1]: *in vitro* cultivated tumor cells were washed three times in HBSS and injected into the shaved flank of Nembutal-anesthetized animals (50 μg/g of body weight). Injections were carried out under a stereoscopic microscope and the volume (i.e., the actual cell number) estimated according to the size of the resulting bleb (approximately 10 μl, equivalent to 10^4 cells per animal). Tumor development was followed by measuring the diameters with calipers three times per week.

Adoptive transfer of thymus cells

Thymus glands were removed from tumor-bearing animals, cleaned from surrounding tissue, teased in HBSS, pooled, counted and injected i.v. or i.p. in a volume of 0.2 ml of HBSS into untreated DBA/2 hosts 18–24 hr before the tumor challenge with 10^4 P-815 cells.

Administration of cyclophosphamide

Cy (Endoxan, Asta-Werke, Brackwede, Germany) was dissolved in sterile PBS and injected i.v. or i.p. before or after challenging with tumor cells.

Statistical

Statistical evaluation was performed using Student's *t*-test.

RESULTS

Effect of adoptively transferred thymus cells on the tumor development

In a first experiment thymus cells were transferred from animals which were injected 8 days before with 10^4 P-815 cells as tumor hosts were shown to bear suppressor cells in their thymus at that moment [3]. Control experiments included transfer of normal thymus cells or HBSS alone. Thymus cells from each group (7 normal animals and 7 tumor bearers) were pooled, washed, suspended in HBSS and injected i.p. into a group of 6 untreated animals which were challenged on the next day with 10^4 P-815 cells i.d. One aliquot of the thymus cell suspension from tumor-bearing animals was in-

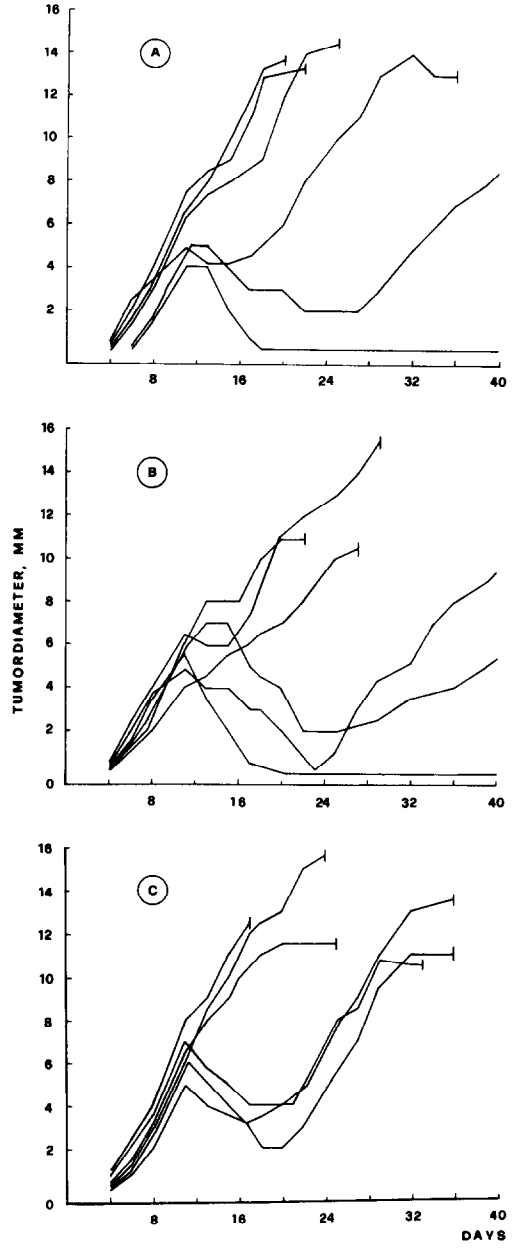

Fig. 1. Tumor development after the adoptive transfer of thymus cells from tumor-bearing animals. Cells from 1 whole thymus i.v. transferred per animal 24 hr before challenging i.d. with 10^4 P-815 cells. Tumor diameters (mm) indicated for individual animals. (A) HBSS alone; (B) thymus cells from normal animals (35.7 × 10^6 per animal); (C) thymus cells from tumor bearers (day 8; 50.6 × 10^6 per animal).

jected i.p. into two animals without challenging in order to confirm the absence of metastatic tumor cells (it is known that 10 P-815 cells i.p. will kill approximately 80% of the animals [1]). Tumor development is shown in Fig. 1 for individual animals in each group. It is evident that there are no major differences in the tumor growth patterns between the 3 groups. Tumors were first measurable around day 6 and reached a first maximum size around day 11. The characteristic phases of regression in some of the animals were observable in both the experimental and the control groups. It is true, however, that the 2 surviving animals were from the control groups (one in each), whereas all animals in the experimental group were eventually killed. There may also be a tendency toward earlier appearance of tumors and less pronounced regression phases in animals transferred with tumor-bearer thymus cells, and more pronounced regressions in the group transferred with an additional normal thymus.

In further experiments it was tested if i.v. instead of i.p. transfer led to more pronounced effects or if cells from days 6 or 10 were more active. As shown in Table 1, this was not the case. The transfer of cells from day 8 tumor bearers results in the largest tumors and the transfer of normal thymus cells in the smallest, when measured on day 11 (Table 1, Exp. 2). The difference, however, could not be established statistically and the survival times of animals succumbing to the tumor also did not show statistically significant differences.

Experiment 3, with transfer of cells from day 6, showed the smallest difference in tumor size and survival times with again one single surviving animal in a control group. Transfer of cells from day 10 (Table 1, Exp. 4) led to smaller tumors than transfer of normal or no thymus cells, but differences were again not statistically significant and survival times did not differ. All 4 experiments together point to a tendency that on day 6 thymus cells are inert and on day 8 they have a rather supressive capacity, whereas on day 10 the effect is rather protective. Transfer of a whole additional normal thymus slightly improved the host's immune defense.

Effect of cyclophosphamide pretreatment on tumor development

Doses of 125, 25 and 5 mg/kg of Cy were administered i.v. two days before a tumor challenge of 10^4 P-815 cells. The highest dose was found by Röllinghoff *et al.* to be most effective in converting low responsiveness to TNP-derived syngeneic cells to high responsiveness [5] and by L'Age-Stehr *et al.* in induction of autoreactivity [8], whereas Askenase *et al.* found that 20 mg/kg affected suppressor cells whilst being without effect on antibody-forming B cells [9].

In analogy to the work of Zatz *et al.* [6] and Tilkin *et al.* [7], modulation of the immune response was also attempted by treating the tumor hosts with Cy on days 6 or 8 after tumor cell injection. Day 6 was demonstrated to represent a period sensitive for affecting sup-

Table 1. *Effect of adoptively transferred thymus cells from tumor-bearing animals on the development of P-815 tumors in DBA/2 mice**

Experiment	Type of thymus	Number of cells per animal ($\times 10^6$)	Tumor diameter (mm) on day 11	Days of survival	Surviving animals
	Tb†, day 8	50.6	6.4 ± 0.42‡	28 ± 3.3‡	0
No. 1	normal	35.7	5.5 ± 0.36	36 ± 4.4	1
i.p. transfer	no	—	5.8 ± 0.53	30 ± 4.9	1
	Tb, day 8	48.0	6.4 ± 0.35	30 ± 2.1	0
No. 2	normal	35.3	4.8 ± 0.60	28 ± 2.3	0
i.v. transfer	no	—	5.7 ± 0.60	35 ± 4.2	0
	Tb, day 6	40.1	5.2 ± 0.10	33 ± 2.1	0
No. 3	normal	31.1	5.3 ± 0.18	31 ± 1.9	0
i.p. transfer	no	—	5.5 ± 0.17	32 ± 3.2	1
	Tb, day 10	68.4	5.3 ± 0.14	32 ± 2.3	0
No. 4	normal	33.1	5.6 ± 0.30	33 ± 1.9	1
i.p. transfer	no	—	5.9 ± 0.16	32 ± 4.8	0

*Transfer of thymus cells 24 hr before challenge with 10^4 P-815 cells i.d., a cell number which induced tumors in all animals. Cells from 1 whole thymus/animal, 6 animals/group.

†Tb: Tumor bearer.

‡$\bar{x} \pm$ S.E.M.

Table 2. Effect of cyclophosphamide treatment of DBA/2 mice on the intradermal development of P-815 tumors

Cyclophosphamide (mg/kg)	Day	Tumor diameter (mm) Day 11	Tumor diameter (mm) Day 15	Days of Survival	Surviving animals
0	-2	5.7 ± 0.62*	5.7 ± 0.63	38 ± 2.7	0
5	-2	5.9 ± 0.58	6.2 ± 0.96	30 ± 1.8	0
25	-2	5.2 ± 0.77	6.3 ± 1.5	——	1
100	-2	5.6 ± 0.45	8.8 ± 0.38	25 ± 1.3	0
0	6	5.5 ± 0.29	7.2 ± 0.94	——	1
50	6	2.9 ± 0.29‡	5.1 ± 0.48§	——	1
0	8	5.6 ± 0.38	6.5 ± 0.97	——	1
50	8†	5.0 ± 0.34	6.2 ± 0.44	——	1

10^4 P-815 cells/animal, 6 animals/group.
*$\bar{x} \pm$ S.E.M.
†Twelve animals/group.
‡$P < 0.01$.
§$P \leq 0.05$.

pressor T cells [7] and day 9 was in the MOPC-315 tumor model a period when administration of Cy induced an otherwise missing phase of tumor regression. The reason for choosing day 8 instead of day 9 in the P-815 model was the onset of tumor dissemination by day 9 (M. Bertschmann, unpublished results).

As summarized in Table 2, pretreatment on day -2 with either dose did not result in better protection of the tumor hosts. In contrast, the group with the highest Cy dose had the largest tumors on day 15 (not on day 11) as a result of missing regression phases. Accordingly, the mean survival time was the shortest in this group. Treatment with lower doses of Cy also resulted in larger tumors and shorter survival times, but differences were not statistically significant from the control group. A single, spontaneous regression in the 25 mg/kg Cy group was within the range of normal variation. Treatment after priming with tumor cells on day 6 resulted in smaller tumors on days 11 and 15, with P values of <0.01 and ≤ 0.05 respectively. The development of tumors after this treatment is illustrated in Fig. 2. It shows that Cy delayed the tumor growth rather

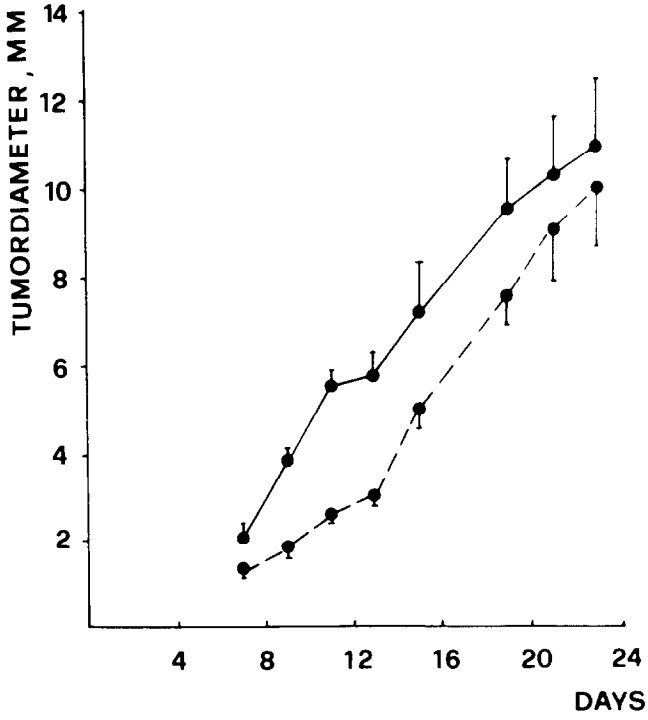

Fig. 2. Tumor development after Cy treatment of tumor hosts: 50 mg CY/kg injected i.p. on day 6 after the i.d. injection of 10^4 P-815 cells ●----●; controls ●——●. S.E.M. indicated.

than resulting in a more pronounced regression phase. The number of surviving animals was the same in all groups.

DISCUSSION

The results presented in this study suggest that excessive activity of suppressor T cells may not be responsible for the untimely termination of the successfully initiated T cell-dependent regression phase, which is a characteristic feature of i.d. P-815 tumor development. This tumor host system seemed to be an especially well-suited model for the modulation of the immune response, since within one experimental group complete spontaneous regressions, transient regressions or growth inhibition and virtual lack of tumor regression can be observed. Nevertheless, adoptively transferred thymus cells from tumor-bearing animals were not capable of abrogating the tumor regression phase, nor was it possible to elevate the number of permanent regressors by treatment with a single dose of Cy injected before or after tumor cell injection.

These findings are in some contrast to other studies in which a major role in progressive tumor development has been attributed to the activity of suppressor cells [10–15]. They are, however, not completely unexpected, as it was previously shown that in the P-815/DBA/2 system the net effect of an adoptive transfer of spleen cells from tumor bearers was protection, not suppression, and a concomitant immunity lasting from days 6–15 with a peak reactivity between days 9–12 was also demonstrated in the same i.d. tumor system [1].

On the other hand, Takei *et al.* [3] have shown, by studying s.c. tumor development, that the P-815 tumor cell is capable of stimulating suppressor cells in the syngeneic host. The most active suppressor T cell population was found by these authors in the thymus of tumor bearers 8 days after the s.c. inoculation of tumor cells. This is shortly before the onset of a transient regression phase which, in contrast to our P-815 line, was observable during s.c. tumor development. Despite the comparable tumor behavior, a discrepancy between the poor reactivity *in vivo* in our studies and the *in vitro* effect as shown by Takei *et al.* [3] is apparent. It should be borne in mind, however, that in Takei's experiment only partial suppression was achieved and the net effect was stimulation of cytotoxic T cells, not abrogation of reactivity.

It was impossible with our P-815 line to also test for the *in vivo* suppressor activity of spleen cells after the conversion from regressive to progressive tumor development, as the complete separation of tumor- from host cells proved to be impossible. The *in vivo* transfer of single, contaminating tumor cells would obscure the results, considering the extremely high malignancy of our P-815 line when injected i.p. [1]. Takei *et al.* [3] were able to demonstrate suppressor cell activity during the corresponding stage of progressive tumor development in their P-815 system, but in this case the spleens were virtually free of metastasizing tumor cells. In other studies emphasizing the stimulation of suppressor cells, the influence of these cells on the tumor development *in vivo* was only transient and rather modest compared to the impressive activity which these cells were able to produce in various *in vitro* tests [11, 12, 15].

Treatment with Cy shortly before the tumor challenge in order to affect suppressor T cells selectively was unsuccessful with all three doses tested. Less pronounced regression phases and accordingly shorter survival times resulted from the treatment, at least with higher doses (125 mg/kg), although this dose and treatment before antigen stimulation was optimal in most other studies [5, 8, 16] and the lowest dose (5 mg/kg) was also shown to be effective [5]. Toxicity of Cy cannot be excluded as a cofactor in the shortening of the survival time although 125 mg/kg of Cy alone did not kill any of the animals (data not shown).

Several authors have shown that the immune response is susceptible to manipulations affecting the suppressor T cell population after priming with tumor cells [6, 7, 13]. The P-815 tumor maintained in this laboratory, however, appeared again to be relatively resistant to Cy treatment during tumor development. It is only after Cy injection on day 6 that the reduction in tumor size was statistically significant during a short period of time. Treatment on day 8 reduced the tumor size to a statistically nonsignificant degree. The tumor growth curves shown in Fig. 2 additionally suggest that the observed delay in tumor growth could as well be due to a direct tumoricidal effect of the drug as to an effect via the host's immune system. The failure to improve tumor repression may partly be explained by the impossibility of exact timing when a growing tumor cell is given as an antigen. Dependence of the effect of Cy on the antigen dose was demonstrated by Askenase *et al.* [9], and L'Age-Stehr *et al.* [8] have shown that pretreatment of animals with Cy results in autoreactive lymphocytes (inhibition of suppressor cells) 6 days later, followed by sup-

pressor cells counteracting autoreactivity. In Röllinghoff *et al.*'s studies [5] the period of enhanced cytotoxicity lasted only for 4–5 days, which may be too short to combat a fast-growing tumor successfully.

Technical reasons may also be responsible for the relative inefficiency of the adoptive transfer of thymus cells from tumor bearers. For example, the number of transferred cells could have been inadequate or the life span of suppressors, suppressor inducers or their precursors [18] may have been too short. Rao *et al.* suspected that the adoptive transfer of Lyt-2 suppressors was an inadequate method of demonstrating their presence because they are not autonomous, requiring other host T cells to induce their activity [18]. Such cooperating cells may not have been available in the new host in adequate numbers or at optimal times.

Hellström *et al.* [19] point to the relationship of immunogenicity of a tumor and its capacity to respond to X-irradiation (used as a method to eradicate suppressor cells). Impressive effects were only obtained with highly immunogenic tumors. A weak effect of suppressor cells would therefore be in agreement with the weak immunogenicity of the P-815 tumor.

In summary we would like to stress the point that we do not claim to have demonstrated the absence of suppressor cells during the immune defence against i.d. developing syngeneic P-815 tumors. As they seem to be a normal component of the regulatory network in every immune response, they may be stimulated during immune reactions against tumors as well. Results obtained by Hellström *et al.* [20] with a Moloney sarcoma virus-induced tumor illustrate the situation: they found the most active suppressor cell population not in tumor bearers but in animals which had spontaneously rejected their sarcomas. These and our own results allow the conclusion that the contribution of suppressor T cells to the subversion of the host's immune reaction by certain tumors is rather limited. Although in many tumor models manipulation of immunoreactivity via the suppressor T cell population seems to be very successful, other tumors may be subversive to the host's defence by other mechanisms, both immunological and non-immunological.

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