

## Effects of isolated tumor lymphocytes alone and with adherent cells

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**Summary.** *The effect on the growth of gradient-isolated mouse mammary tumor cells of different populations of lymphoid cells were evaluated in microtoxicity assays. Variable effects were obtained with tumor-bearer lymph node and spleen cells: in some experiments growth stimulation occurred, whereas in others inhibition was observed. Mixed effector populations gave more regular results: adherent spleen cells added to lymph node or spleen lymphocytes inhibited tumor cell growth in six of nine experiments; inhibition occurred when either of the effector populations in the mixture was derived from the tumor-bearing mouse. Tumor-associated lymphoid cells (TAL) stimulated growth of the tumor cells in five of seven experiments. However, TAL inhibited tumor growth when combined with adherent spleen cells from tumor-bearing animals. In contrast with the peripheral lymphoid cells, admixture of control adherent cells from normal animals with TAL did not inhibit growth. No natural killer effect was seen in these growth inhibition assays. These data indicate that lymphoid populations capable of inhibiting tumor cell growth can be found in tumor-bearing animals, but such combination of active cells are not present at the tumor site.*

### Introduction

Previous publications have described the isolation, characterization, and functional activities of lymphoid cells from mouse mammary tumors [3–6]. In the studies gradient-separated TAL (tumor-associated lymphocytes) were used in assays with tumor cells grown in cell culture for the 7 days preceding experiments TAL were found to markedly stimulate tumor cell growth in microcytotoxicity [5] and Winn [6] assays. Treatment of the TAL with antilymphocyte serum and complement abrogated their stimulatory effects in both in vitro and in vivo assays. TAL were also found to be unresponsive in blastogenesis assays with either PHA or soluble tumor extracts [6].

Our recent development of the methodology for the simultaneous gradient separation of fresh mammary tumor cells and TAL [7] makes it possible to study the activity of isolated cells from individual autochthonous mouse mammary

tumors. The present study describes the effects on tumor cell growth in vitro of TAL and lymph node and spleen cells from the tumor bearer alone, and in combination with adherent spleen cells.

### Materials and Methods

**Tumors.** Autochthonous mammary tumors arose spontaneously in strain C3H/He female mice. This strain was obtained in 1950 from T. Hauschka and maintained in the Department of Tumor Biology at the Karolinska Institute by brother-sister matings.

**Isolation of tumor and lymphoid cells.** The details of this preparation have been described elsewhere [7]. Briefly, tumors were passed through sterile steel mesh and then enzymatically digested with a combination of collagenase and DNAase. The enzymatically digested tumor suspension was layered over a 50% FCS gradient and sedimenting cells were collected, filtered, and used as target tumor cells.

**Lymph node and spleen cells.** Lymph nodes (LN) and spleens (S) were teased with needles to obtain suspensions. The spleen suspensions were treated with hypotonic lysis to eliminate erythrocytes. The cells were washed twice in medium, counted, and adjusted to the appropriate concentration. Suspensions were made of control (C) and tumor bearer (TB) cells.

**Adherent spleen cells.** Cell suspensions obtained from minced spleens of control and tumor-bearing animals were incubated in plastic flasks for 30 min at 37° C. The nonadherent population was collected. The adherent populations (SA) were washed and detached from the plastic surface by incubation with 2 mM EDTA for 15 min at 37° C followed by scraping with a rubber policeman. These cells were washed, resuspended, and used as the adherent cell population in the microcytotoxicity assays. Usually better than 95% of the cells were viable as indicated by trypan blue exclusion.

**Plating of tumor cells for the microcytotoxicity assay.** The freshly isolated, filtered tumor cells were resuspended to a concentration of  $5 \times 10^5$  cells/ml in RPMI with 10% FCS. Three serial dilutions of this suspension were seeded in 20- $\mu$ l aliquots into Falcon microtest plates. After incubation overnight at 37° C, the plates were scored for the number of cells. A variation was found in the plating efficiency of the cells isolated from

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**Table 1.** Summary of the effect of lymph node and spleen cell populations on the in vitro growth of mammary tumor cells

Effector populations <sup>a</sup>	Number of experiments and mice	Effects on tumor cell growth in vitro					
		Absolute change <sup>b</sup>			Relative change <sup>b</sup>		
		Increased	None	Decreased	Increased	None	Decreased
1. CLN	12	5	7	—			
2. CS	7	6	1	—			
3. CSa	9	2	6	1			
4. TBLN	12	5	4	3	6	3	3
5. TBS	7	4	3	—	2	4	3
6. TBSa	7	5	4	—	4	3	2
7. TAL	7	5	1	1	5	1	1

<sup>a</sup> C, control; TB, tumor bearer; LN, lymph node; S, spleen; Sa, adherent spleen cells; TAL, tumor-associated lymphocytes

<sup>b</sup> Absolute change is evaluated in relation to control wells without effector lymphocytes. Relative change is evaluated in relation to wells with control reflector lymphocytes at 40 : 1 effector target ratios after 48 h incubation. Results were evaluated by the Mann-Whitney nonparametric test

**Table 2.** Selectivity of action by the lymphoid populations of the tumor bearer

Effector populations <sup>a</sup>	Experiments	No. of animals with selective action <sup>b</sup>
1. TBLN	9	3
2. TBS	8	3
3. TBSa	9	7
4. TAL	9	2

<sup>a</sup> 40 : 1 effector-to-target ratio

<sup>b</sup> An effect is judged as selective when it occurred with the autologous tumor but not with another syngeneic tumor. Significance of difference in inhibition or stimulation compared with control was judged by the Mann-Whitney nonparametric test

different tumors (range 5%–55%). Plates were inspected and the dilution containing 100–200 attached tumor cells was selected for the microcytotoxicity assay.

**Microcytotoxicity assay.** The microcytotoxicity assay was performed according to the method of Takasugi and Klein [22]. Isolated tumor cells were seeded as indicated above into assay plates. After 24 h the medium and nonadherent cells were decanted and 20  $\mu$ l effector cells was added to each well to give 40 : 1 and 20 : 1 effector-to-target ratios. Replicates of two to eight wells were used per sample. The plates were incubated for another 48 h at 37° C. At the end of incubation, they were washed three times with saline, fixed, and stained with May-Grunwald-Giemsa stain, after which the number of cells remaining in the wells was counted. Data were evaluated by the Mann-Whitney nonparametric test.

## Results

### *Effect of lymphoid cells on the in vitro growth of tumor cells*

Isolated tumor cells obtained from velocity gradients were seeded into microcytotoxicity plates as described elsewhere [7]. After 24 h the nonadherent tumor cells were removed and lymphocyte effectors were added to the individual culture wells. Lymph node, spleen and TAL suspensions were added

to tumor cell cultures in experiments with 12 different primary tumors. A summary of data evaluations is presented in Table 1. Experiments were performed at 40 : 1 and 20 : 1 effector-to-target ratios. The data are presented for the 40 : 1 test since similar but smaller differences were found at the 20 : 1 ratio. Lymphoid cells from non-tumor-bearing syngeneic animals frequently had a feeder effect on tumor cell growth, increasing for example from 218 to 270 cells. Stimulation above control levels occurred with six of twelve tumor-bearer (TB) lymph node and with two of seven TB spleen cells. Three of the twelve TB lymph node and three of the seven TB spleen cells had inhibitory effects compared with control cells, decreasing for example from 95 to 75 cells. Adherent cells from TB spleen were tested in nine cases: they stimulated growth in five and inhibited it in four. TAL were stimulatory to tumor cell growth in five of seven and inhibitory in one of seven experiments. TAL was the only cell population that showed regularity in its effect, in that it stimulated growth in the majority of the experiments. The results of experiments with TAL were controlled for the low numbers of contaminating tumor cells which may have been present by subtracting the number of cells present in wells with TAL alone (wells with TAL alone averaged 27 tumor cells/4  $\times$  10<sup>4</sup> TAL and 50 tumor cells/8  $\times$  10<sup>4</sup> TAL). Increased numbers of tumor cells were frequent in wells that had effector populations of TAL, lymph node, or adherent spleen cells from the tumor-bearing animals.

To reveal a possible tumor selectively, lymphoid cell populations were also tested for effects on the growth of cells from an independent syngeneic tumor (Table 2). TB lymph node, spleen, and TAL usually affected the growth of autochthonous and syngeneic tumor cells similarly. Only the adherent spleen cells showed selective effects in the majority of experiments, frequently enhancing but occasionally reducing tumor cell growth.

### *Effect of adherent spleen (Sa) cells acting in combination with other lymphoid populations*

Growth was inhibited by combinations of adherent cells, derived from tumor bearer spleens, with TB lymph node or nonadherent spleen cells. Two representative experiments are presented in detail (Tables 3 and 4). In one experiment (Table 3) all control effectors had some stimulatory effect but the TB lymph node cells stimulated even more substantially

**Table 3.** The growth of mammary tumor cells in the presence of mixed lymph node and adherent spleen cell populations

Effector populations	Target cells			
	Tumor 1		Tumor 2	
	Number of target cells/well after 48 h <sup>b</sup>	% Change (relative) <sup>c</sup>	Number of target cells/well after 48 h <sup>b</sup>	% Change (relative) <sup>c</sup>
1. (Medium control)	218 ± 14.2		186 ± 9.6	
2. CLN	270 ± 16.6		176 ± 10.1	
3. CSa	230 ± 15.3		180 ± 8.9	
4. CLN + CSa	290 ± 16.3		210 ± 11.2	
5. TBLN	380 ± 18.5	+ 41*	240 ± 13.4	+ 36*
6. TBSa	275 ± 15.5	+ 20*	175 ± 8.4	- 3
7. TBLN + TBSa	130 ± 9.2	- 54*	155 ± 7.2	- 27*
8. TBLN + CSa	180 ± 10.1	- 38*	160 ± 7.6	- 24*
9. CLN + TSa	155 ± 9.3	- 46*	165 ± 7.8	-21*

<sup>a</sup> 40 : 1 effector-to-target ratio of cells from tumor bearer no. 1

<sup>b</sup> Mean ± SE

<sup>c</sup> Relative change is evaluated in relation to the wells with effector lymphocytes from the same source in the control. Mixed populations are evaluated in comparison with the population on line 4, i.e., CLN and CSa

\* Significant difference ( $P < 0.05$ )

**Table 4.** The growth of mammary tumor cells with presence of mixed spleen and adherent spleen cell populations

Effector populations	Target cells			
	Tumor 3		Tumor 4	
	Number of target cells after 48 h <sup>b</sup>	% Change (relative) <sup>c</sup>	Number of target cells after 48 h <sup>b</sup>	% Change (relative) <sup>c</sup>
1. (Medium control)	55 ± 5.5		95 ± 7.1	
2. CS	95 ± 6.9		120 ± 8.5	
3. CSa	85 ± 6.3		160 ± 9.7	
4. CS + CSa	85 ± 5.9		90 ± 6.8	
5. TBS	75 ± 5.7	- 21*	115 ± 7.2	- 4
6. TBSa	105 ± 8.1	+ 23*	165 ± 10.1	+ 3
7. TBS + TBSa	65 ± 6.1	- 24*	90 ± 7.4	0
8. TBS + CSa	60 ± 5.7	- 30*	100 ± 6.8	+ 11
9. CS + TBSa	60 ± 5.9	-30-	105 ± 7.2	+ 16

<sup>a</sup> 40 : 1 effector-to-target ratio of cells from tumor bearer no. 3

<sup>b</sup> Mean ± SE

<sup>c</sup> Relative change is evaluated in relation to the wells with control lymphocytes

\* Significant difference ( $P < 0.05$ )

**Table 5.** Summary of results of individual experiments with combinations of lymph node and adherent cell populations

Effector populations <sup>a</sup>	Change relative to	% Change <sup>b</sup>										Experiments showing change		
		1	2	3	4	5	6	7	8	9	10	Increase	None	Decrease
1. TBLN + CSa	CLN + CSa		-18		-48	-20	-18	-16	0	+21	0	1	2	5
	TBLN	-63	-23		-18	0	-38	+24	-7	+21	-15	1	2	6
2. TBLN + TBSa	CLN + CSa		-23	-40	-56	-20	-18	-26	0	+21	- 0	1	2	6
	TBLN		-32	-39	-31	0	-38	-26	-4	+21	-15	1	2	6
3. CLN + TBSa	CLN + CSa		-25	-53	-39	-15	-15	-14	0	+14	+21	2	1	6
	TBLN		-34	-52	- 5	0	-36	+27	-7	+14	0	2	2	5

<sup>a</sup> 9 experiments evaluated for results at 40 : 1 effector-to-target ratio

<sup>b</sup> Each number represents the results from an experiment with an individual autochthonous tumor in the microcytotoxicity assay evaluated as in Tables 3 and 4

when administered at 40 : 1 or 20 : 1 effector-to-target ratios. Only results obtained with the 40 : 1 ratio are presented here. Whereas tumor-bearer adherent cells were not growth-inhibitory alone, they exhibited a strong inhibitory effect when combined with lymph node cells. Inhibition of growth occurred with the addition of TB adherent cells to lymph node cells from either control or tumor-bearing animals. In addition, mixtures of tumor bearer lymph node and control adherent cells also caused growth inhibition. Such populations reduced the cell number to well below the level of the control cultures to which effectors had not been added. (Compare samples 7, 8, and 9 with sample 1.) Not only was the stimulation abrogated but

there was real growth inhibition. Thus, results with the various mixtures indicate that as long as one part of the mixed population was derived from the tumor-bearing animal, inhibition occurred. (Compare samples 7, 8, and 9 with sample 4.) In another representative experiment (Table 4) with spleen cells, effector populations from control animals enhanced tumor cell growth. However, the TB spleen cells inhibited growth compared with spleen cells from control animals. Nevertheless, the addition of adherent spleen effectors to the lymphoid cells reduced tumor cell growth even further. (Compare samples 7 and 8 with sample 6.) Here again, the results with the mixed population indicate that significant abrogation of growth can occur as long as one component of the combined populations is obtained from the tumor bearer. The summary of results obtained with the mixed effector populations is presented in Table 5. Growth inhibition occurred in six of nine experiments with the TB lymph node populations combined with adherent spleen cells from either tumor-bearing or control animals. Similar results were obtained with TB spleen cells. Adherent cell populations from the tumor bearer also inhibited tumor cell growth when used in combination with control lymph node cells in six of nine experiments. An evaluation of results in experiments with combined populations containing lymph node and adherent cells shows that growth-inhibitory effects were specific for the autologous tumor in the majority of cases (Table 6).

**Table 6.** Selectivity of action of combined populations of lymph node and adherent spleen cells on tumor cell growth

Effectors combined <sup>a</sup>	Number of experiments	Inhibition of tumor cell growth	
		Autologous	Syngeneic
TBLN + CSa	8	6	2
TBLN + TBSa	7	5	2
CLN + TBSa	8	6	2

<sup>a</sup> 40 : 1 effector-to-target ratio

**Table 7.** The growth of mammary tumor cells in presence of combined TAL and adherent cell populations

Effector populations <sup>a</sup>	Target cells					
	Tumor 5			Tumor 6		
	Number of target cells after 48 h <sup>b</sup>	Control for contamination <sup>c</sup>	% Relative change <sup>d</sup>	Number of target cells after 48 h <sup>b</sup>	Control for contamination <sup>c</sup>	% Relative change <sup>d</sup>
1. (Medium control)	92 ± 6.2			58 ± 5.7		
2. CLN	107 ± 7.1			60 ± 5.9		
3. CSa	110 ± 7.3			70 ± 6.2		
4. CLN + CSa	116 ± 6.9			74 ± 6.5		
5. TBSa	105 ± 6.9			100 ± 7.2		
6. TAL	225 ± 10.2	175		210 ± 9.7	160	
7. TAL + TBSa	90 ± 5.9	40	- 77	73 ± 6.1	23	- 86
8. TAL + CSa	216 ± 9.3	166	- 5	13 ± 8.2	133	- 17

<sup>a</sup> Effector-to-target ratio 40 : 1, tumor no. 5

<sup>b</sup> Mean ± SE

<sup>c</sup> Corrected for tumor cell contamination in wells with control TAL:  $8 \times 10^4$  cells/well had 50

<sup>d</sup> Relative change is evaluated in relation to the wells with TAL

**Table 8.** Summary of the effects of combined populations of TAL with adherent cells from controls and autologous tumor bearers on the vitro growth of mammary tumor cells

Effector population <sup>a</sup>	% Change relative to TAL <sup>b</sup>							Comparative results		
	1	2	3	4	5	6	7	Increase	No change	Decrease
1. TAL + CSa	0	0	-32	0	+32	+23	+20	3	3	1
2. TAL + TBSa	-	-21	-28	-10	-53	0	+20	1	1	4

<sup>a</sup> 40 : 1 effector-to-target ratio

<sup>b</sup> Each number represents the results from an experiment with an individual tumor in the microcytotoxicity assay evaluated as in Table 7

### *Effect of TAL in combination with adherent cells*

As described above, isolated TAL were usually stimulatory to the *in vitro* growth of tumor cells (Table 7). A mixture of TAL with adherent cells from the tumor bearer, but not from a control animal, abrogated this stimulatory effect (Table 7: cf. lines 6, 7, and 8). These results contrast with the combined effects of control adherent cells with TB lymphoid cells derived from either lymph node or spleen. (Compare Table 3, line 8; Table 4, line 8; and Table 7, line 8). A summary of seven experiments in which TAL were used with adherent cells is presented in Table 8. Growth inhibition exerted by the mixture of TAL and adherent TB spleen cells occurred in four of six experiments, whereas the mixture of TAL and control adherent spleen cells inhibited growth in only one of seven experiments.

### **Discussions**

We have used gradient-isolated tumor and lymphoid cells from spontaneous tumors to evaluate autologous immunity in the primary tumor-bearing animal. In our previous study with short-term <sup>51</sup>Cr-release assays lymphocyte-mediated cytotoxicity against separated tumor cells was found only occasionally [7]. In these present experiments microcytotoxicity assays were used to measure longer interactions between effector and target cells. In addition to the extension of the interaction, this assay also measures the influence of lymphoid cells on the growth of tumor cells. We consider enhancement of growth, if specific for the system, also to be an important sign of recognition and interaction between the effectors and the targets [18]. In our studies with individual lymphoid cell populations growth stimulation was frequently observed, whereas growth inhibition occurred only occasionally. However, mixtures of adherent cells derived from spleens with lymph node (or nonadherent spleen) cells were growth-inhibitory when either of the two components entering in the mixture was derived from a tumor-bearing animal. Therefore, an interaction between different lymphoid populations was required to exert the antitumor effect. No control lymphoid cell populations inhibited tumor cell growth when acting alone. Thus, as with the short-term assays [7], no natural killer (NK) effect was found against these freshly isolated tumor cell targets in the microcytotoxicity studies. Results from other laboratories using targets from cell lines indicate that cells with NK activity may be present *in situ* within primary murine tumors [11, 12].

These experiments confirm previous findings with mammary tumors of BALB/cf3H mice [5], in that TAL stimulated the growth of the tumor cells. Since the evaluation was based on the number of cells in the wells, and the TAL population contained a small number of tumor cells, every TAL population had to be cultured alone and controlled for cell contamination. The number of contaminating tumor cells could not account for the enhanced number of tumor cells in the cytotoxicity assays. TAL populations initially contained few monocyte-like cells (range 2%–6%) following gradient separation, and even fewer (<1%) following adherence to remove tumor cell contamination [7]. As with previous studies [5, 6] TAL often differed from lymph node and spleen cells in their effect on tumor cell growth. In the present study we found that addition of adherent cells from the spleen of the animal bearing the same tumor to the TAL resulted in growth inhibition. Note that while the lymph node cell population combined with adherent spleen cells inhibited tumor cell

growth irrespective of their origin, as long as either one was derived from the tumor bearer, TAL inhibited only when autologous adherent spleen cells were also present. In previous studies TAL from Balb/cf3H tumors did not respond in blastogenesis assays and suppressed the response of normal lymphocytes to PHA [6].

Experiments with tumor-associated macrophages suggest that these cells may also play a role in regulating tumor growth [8, 9, 15, 20, 23]. Poor responsiveness to antigenic stimulation has been reported in the monocyte-macrophage-like cells of both human and animal tumor bearers [1, 2, 10, 16, 17, 21]. Studies by Johnson et al. have suggested that the growth of large tumors may be the result of a shortage of macrophage effector cells within the tumor site rather than the result of a systemic deficiency of these cells [13]. Moreover, elutriation analysis of murine mammary tumor-associated macrophages indicates that metastatic and nonmetastatic mammary tumors differ in the distribution and functional capacity of their macrophage subpopulations [14]. The immune response involves the interaction of many lymphoid subpopulations, mediated in part by the action of soluble factors, lymphokines. In addition, mouse mammary tumors with different biological properties have been shown to differ in the distribution of TAL Lyt subsets [19]. The small numbers of lymphoid cells found within and around these murine adenocarcinomas indicate that few accessory mononuclear cells are chemotactically attracted to the tumor site. The results of our *in vitro* tests suggest that lymphoid populations that exert inhibitory effects on tumor growth can be found in the tumor-bearing animal. However, such cells are not present in active cellular combinations at the tumor site. Whether this is the result of an effect or effector cell traffic into, or out of, the tumor site, or to an impairment of effector cell function is not known.

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