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Characterization of Tumor-Associated Lymphocytes in a Series of Mouse Mammary Tumor Lines with Differing Biological Properties

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Summary. *Tumor-associated lymphocytes were isolated by isokinetic gradient separation from five related mouse mammary tumor lines with different immunological and growth characteristics. Although considerable variation in recovery rates was seen from experiment to experiment, the five tumor types were found to have reproducible and characteristic patterns of T lymphocyte subpopulations, as detected by cytotoxicity assay using monoclonal antisera to Thy-1, Lyt-1, and Lyt-2 antigens. Tumors of line 168, which are weakly immunogenic at best, had the lowest numbers of recovered ALS^+, Thy 1^+ lymphocytes (12% and 9%, respectively), in contrast to immunogenic lines (mean 38% and 26%, respectively). Line 68H tumors, which grow after prolonged latency periods and also produce tumor cell variants in vivo, were unique in that the numbers of recovered Lyt 1^+ lymphocytes exceeded the numbers of Lyt 2^+ lymphocytes, whereas these two T cell subpopulations were either equal or Lyt 2^+ cells predominated in the other faster growing, non-variant-producing tumors. No differences in T lymphocyte distribution were associated with the presence or absence of metastatic behavior. These results indicate that distinctive lymphocyte infiltrates may be characteristic of tumors with distinct biological differences.*

Introduction

Previous reports from our laboratory have described isokinetic gradient separation of infiltrating host cells from solid mouse mammary tumors [1]. The relative distribution of infiltrating cell types was found to vary reproducibly among independently arising tumors, suggesting that the type of infiltrate is a tumor, not a host, characteristic [2]. In our studies the lymphocyte component was either T or 'null' cell; surface immunoglobulin-bearing cells were rare. The aims of the present study were to extend the characterization of tumor-associated lymphocytes by utilizing monoclonal antibodies against T cell subpopulations and to compare the T cell subpopulation distribution in a series of mammary tumor lines that differ in immunological and growth properties and in ability to metastasize from a primary implant.

Materials and Methods

Mice. Male BALB/cfC_3H mice were purchased from the Cancer Research Laboratory, University of California at Berkeley, Berkeley, California, USA.

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Tumors. All tumor lines were derived from a single, spontaneously arising mammary tumor of a strain BALB/cfC_3H mouse. Lines 68H and 168 were derived in vitro from the original tumor [5]. Line 168 was further cloned in soft agar. Lines 410, 410.4, and 4501 were all derived from a single metastatic nodule in the lung of a BALB/cfC_3H mouse carrying the tenth SC in vivo passage of the parent tumor [8]. Line 410 is a relatively non-metastatic variant which arose after 12 in vitro passages. Cells from the original lung nodule were also serially transplanted through four passages in vivo, at which time the tumor cells were reestablished in tissue culture and designated 410.4. Line 4501 was derived by dilution cloning of a cell suspension made from a 410.4 tumor growing in the subcutis. Both 410.4 and 4501 are highly metastatic. Mammary tumors were passaged by SC injection of 10^6 trypan blue-excluding tumor cells into syngeneic mice. The various characteristics of the tumor subpopulations are given in Table 1.

Cell Preparations. Tumors were minced into 1–2 mm pieces and digested with collagenase (250 U/ml, Worthington Biochemical Corp., Freehold, NJ, USA) and deoxyribonuclease (1 mg/ml Sigma Chemical Co., St Louis, MO, USA) for 60 min at 37° C at a concentration of 25 ml/g tumor tissue. The suspension was pipeted vigorously, filtered through 150 μm Nytex, and washed three times at 2,000 rpm.

Table 1. Characteristics of tumor lines from a single BALB/cfC_3H mammary adenocarcinoma

Subpopulation	Mean tumor[a] free days	Spontaneous[b] metastasis	Immune profile		
			Primary[c]	Secondary[d]	Sensitivity[e]
68H	60	0/8	++	–	+
168	19	2/60	±	–	+
410	24	3/36	++	++	++
410.4	14	36/44	ND	+	+
4501	14	41/62	ND	–	+

[a] After an inoculum of 10^5/cells
[b] Number of animals with lung metastases/total number of animals from which tumors injected SC were surgically removed
[c] Based on relative growth in normal mice versus growth in adult mice immunosuppressed by 400 R irradiation 2 days prior to injection ± thymectomy
[d] Ability to induce transplantation immunity, i.e., resistance to implanted cells approximately 10 days after surgical removal of tumor
[e] Ability to respond to transplantation immunity

Cells were counted in a hemacytometer and diluted for layering on a isokinetic gradient in RPMI 1640 medium (Grand Island Biological Co., Grand Island, NY, USA) and 2% newborn calf serum.

Isokinetic Separation. Isokinetic separation was accomplished with the continuous gradient method of Pretlow [12]. Gradients were prepared in a commercially available gradient maker (Lido Glass Co., Sterling, NY, USA) that was modified by replacing the stopcock plug (No. 7681-2 Corning Glass Works, Corning, NY, USA). A continuous gradient was formed of Joklik's MEM and Ficoll (Sigma, St Louis, MO, USA) in Joklik's MEM, at a refractive index of 1.3435. Five percent CO_2 was bubbled into the Ficoll through a 0.22-μm Millipore filter. A cushion of 5.5 ml 43% Ficoll was placed in the bottom of siliconized no. 2086 IEC centrifuge tubes. A maximum of 3×10^7 cells in 7 ml Joklik's modified MEM with 10% fetal calf serum was layered over the gradient. Centrifugation was in an IEC 6000 centrifuge for 14 min at 4° C at 97 *g* measured at the sample-gradient interface. The fractions were collected with a tapping cap (Halpro, Inc., Rockville, MD, USA) by displacement with an iced 55% sucrose solution. Gradients were collected as 12 fractions, the first containing 11 ml, the others 8 ml. The fractions were diluted with Joklik's modified MEM and centrifuged in an IEC 6000 centrifuge at 125 *g* for 15 min. The cells were resuspended in medium and counted in a hemacytometer.

Antisera and Cytotoxicity Test. The various cell populations were assayed with monoclonal antisera (New England Nuclear, Boston, Mass.) against Thy 1.2, Lyt 1.2, and Lyt 2.2 antigens and with antilymphocyte serum (ALS). RPMI 1640 medium and 2% newborn calf serum was used throughout. Antisera in 50-μl volumes at 1 : 100 dilution were added to 50-μl aliquots of test cells in a concentration of 5×10^6/ml and incubated for 30 min at 37° C. Rabbit complement (Cedarlane Laboratories, Westbury, NY) was added at a 1 : 20 dilution in 50-μl volumes, followed by incubation for an additional 30 min at 37° C. Trypan blue was added at a 1 : 4 dilution in 50-μl volumes and the percentage of viable dead cells scored. Percent cytotoxicity was calculated as follows:

$$\%\ \text{Cytotoxicity} = 100 \times \frac{\%\ \text{dead (antibody plus complement)} - \%\ \text{dead (complement alone)}}{100 - \%\ \text{dead (complement alone)}} .$$

Results

Table 2 presents results of eight isokinetic gradient separations of tumors of subline 68H. Considerable quantitative variation is evident between experiments. Monoclonal antibody analysis was confined to fractions 4 and 5, the peak lymphocyte fractions. The percentage of cells recovered in fraction 10, the peak tumor cell-containing fraction, is presented also. The percentage of total cells recovered from the gradient ranged from 41% to 76%. Line 68H tumors are difficult to disaggregate into single cells and are quite prone to clump after disaggregation. Clumped cells go to the bottom of the gradient and are lost to further analysis. The percentage of total recovered cells in fractions 4 and 5 ranged from 29% to 56%. Of these cells 74%–98% were lysed by ALS plus complement and 50%–74%, by anti-Thy 1.2 and complement. Lyt 1.2 antibody delineated 26%–56% and Lyt 2.2 0%–53% of the fractions and cells.

Variation between experiments cannot be ascribed to tumor size or time after tumor injection (Table 2). Nor was sex of tumor host a factor (data not shown). Tumors were grown either in exogenous murine mammary tumor virus (MuMTV)-free BALB/c or MuMTV-carrying BALB/cfC_3H hosts. Similar results were obtained in both.

Isokinetic gradient separations were carried out on tumors from four other sublines: 168, 410, 410.4, and 4501. Mean data

Table 2. Lymphocyte patterns in tumors of subpopulation 68H

	Experiment							
	1	2	3	4	5	6	7	8
Tumor weight (mg)	350	500	500	600	600	700	900	950
Tumor age (weeks)	6	4	5	6	5	5	7	8
Efficiency of disruption[a]	99	72	80	126	81	69	138	101
Percent of total cells recovered[b]	55	46	76	41	64	42	60	64
Percent fraction 4 and 5 of recovered[c]	29	53	34	39	45	51	48	56
Percent fraction 10 of recovered[d]	19	9	16	28	28	27	14	17
Percent of fraction 4 and 5[e] which were								
ALS$^+$	86	98	–	79	74	92	82	83
Thy 1.2$^+$	72	51	–	50	74	71	74	70
Lyt 1.2$^+$	26	26	–	50	46	32	36	56
Lyt 2.2$^+$	14	9	–	0	16	53	19	2

[a] Number of cells ($\times 10^{-6}$) obtained per gram of tumor digested

[b] $\frac{\text{Cells recovered in all fractions}}{\text{Cells applied to gradient}} \times 100$

[c] $\frac{\text{Cells recovered in fractions 4 and 5}}{\text{Cells recovered in all fractions}} \times 100$

[d] $\frac{\text{Cells recovered in fraction 10}}{\text{Cells recovered in all fractions}} \times 100$

[e] $\frac{\text{Marker-positive cells}}{\text{Cells recovered in fractions 4 and 5}} \times 100$

for all gradients are presented in Tables 3 and 4. Tumors of line 168, which are more easily placed into single cell suspension than are the others, gave the best overall cell recovery. The percentage of recovered cells in the lymphocyte fractions was smaller for 168 than for the other four tumors. Variability for all lines was similar to that for 68H tumors.

Inspection of the distribution of T lymphocyte subpopulations in the five different tumors (Table 4) reveals major differences: The lymphocytic infiltrate was less in tumor 168 than in the other tumors; the tumor 68H infiltrates were enriched for Lyt 1.2$^+$ lymphocytes; and the tumor 410 infiltrates were enriched for Lyt 2.2$^+$ lymphocytes. These differences are not due to changes in the lymphocyte distribution on the gradients (Table 5). Infiltrates into both metastatic and non-metastatic variants of 410 were similar. Our results indicate that the pattern of T lymphocyte distribution is characteristic for each tumor line. Additional evidence for this hypothesis was obtained by experiments in

Table 3. Isokinetic gradient separation of five tumor subpopulations

	Tumor subpopulation				
	68H	168	410	410.4	4501
Number of Experiments	18	13	14	3	4
Efficiency of disruption[a]	102 (69–138)[b]	155 (83–179)	102 (59–148)	80 (71–87)	150 (139–172)
% Total recovery[c]	52 (33– 76)	71 (51– 92)	44 (18– 52)	43 (42–46)	50 (43– 57)
Fractions 4 and 5[d]	44 (29– 70)	13 (5– 26)	45 (31– 59)	42 (39–44)	36 (29– 43)
Fraction 10[e]	17 (3– 34)	72 (45– 90)	10 (4– 17)	19 (10–26)	12 (9– 13)

[a] Number of cells ($\times 10^{-6}$) obtained per gram of tumor digested

[b] Mean (range)

[c] $\frac{\text{Cells recovered in all fractions}}{\text{Cells applied to gradient}} \times 100$

[d] $\frac{\text{Cells recovered in fractions 4 and 5}}{\text{Cells recovered in all fractions}} \times 100$

[e] $\frac{\text{Cells recovered in fraction 10}}{\text{Cells recovered in all fractions}} \times 100$

Table 4. Lymphocyte content of tumor subpopulations

Tumor subpopulation	% ALS$^+$	% Thy 1.2$^+$	% Lyt 1.2$^+$	% Lyt 2.2$^+$
68H (13)[a]	36 (25–51)[b]	24 (16–39)	25 (9–46)[d]	8 (0–27)
168 (11)	12 (5–22)[c]	9 (2–18)[c]	4 (0–22)	8 (4–16)
410 (11)	39 (27–58)	29 (21–44)	8 (0–38)	24 (0–48)[e]
410.4 (3)	38 (36–40)	24 (19–28)	0	22 (18–24)
4501 (4)	35 (28–41)	23 (16–32)	4 (3– 5)	22 (16–27)

[a] Number of complete experiments

[b] Mean percentage of marker-bearing cells (range) calculated on basis of total cells recovered from gradients

[c] Marker content of 168 is significantly lower than that of 68H or 410; $P < 0.002$ by Wilcoxin statistic

[d] % Lyt 1.2 of 68H is significantly higher than that of 168 or 410; $P < 0.002$ by Wilcoxin statistic

[e] % Lyt 2.2 of 410 is significantly higher than that of 68H or 168; $P < 0.002$ by Wilcoxin statistic

Table 5. Distribution of lymphocytes in gradient fractions

	Tumor subpopulation				
	68H		168		410
	Expt 1	Expt 2	Expt 1	Expt 2	Expt 1
Fractions 1–3[a]	7	2	0	0	9
Fractions 4 and 5[b]	79	84	100	93	87
Fractions 6–10[c]	14	14	0	7	4

[a] $\frac{\text{ALS}^+ \text{ cells recovered in fractions 1–3}}{\text{Total ALS}^+ \text{ cells recovered in all fractions}} \times 100$

[b] $\frac{\text{ALS}^+ \text{ cells recovered in fractions 4 and 5}}{\text{Total ALS}^+ \text{ cells recovered in all fractions}} \times 100$

[c] $\frac{\text{ALS}^+ \text{ cells recovered in fractions 6–10}}{\text{Total ALS}^+ \text{ cells recovered in all fractions}} \times 100$

Table 6. Isokinetic gradient separation of two tumors on contralateral sides of individual mice

Percent of total cells recovered which were	Group 1		Group 2		Group 3	
	Side 1, 68H	Side 2, 68H	Side 1, 68H	Side 2, 168	Side 1, 168	Side 2, 168
ALS$^+$	31[a]	37	36	14	17	10
Thy 1.2$^+$	24	21	18	9	13	7
Lyt 1.2$^+$	20	14	14	2	8	1
Lyt 2.2$^+$	4	6	4	10	10	4
Lyt 1.2: Lyt 2.2	5	2.3	3.5	0.2	0.8	0.25

[a] Mean of three experiments

which the same mouse was injected on opposite sides with cells from different tumor lines. Isokinetic gradient separations and T lymphocyte characterization were performed on the individual tumors. The results in Table 6 show that the patterns of 68H and 168 were the same when grown concomitantly on the same host as when each was grown separately; the percentage of Lyt 1.2^+ cells was greater than that of Lyt 2.2^+ cells in the 68H tumors, and Lyt 2.2^+ cells equaled or exceeded Lyt 1.2^+ cells in the 168 tumors. Similar bilateral experiments with 410 and 168 tumors also demonstrated the stability of T lymphocyte pattern (data not shown).

Discussion

The long-term goal of our work is the understanding of the role of host immune events in the growth and behavior of cancers. We have focused on the description of the nature and activity of host effector cells that are located within tumor masses since it seems likely that the tumor itself would be a major focus for host immune influence. We have used the isokinetic gradient separation techniques of Pretlow [12] to isolate lymphocytes from solid mouse mammary tumors and have characterized the types of lymphocytes and measured their function [1–4]. In the present study we compared the tumor-associated lymphocytes of tumors produced by five cell lines that differ in growth, metastatic, and immunological properties. Three of these lines were derived from the same strain BALB/cfC_3H mammary tumor. Two additional lines were developed from clones of one of those three lines. By using these related lines, we hope to minimize differences that are not relevant to tumor behavior.

Isokinetic gradient separation is not a precise quantitative technique. The tumor cells are epithelial and are difficult to dissociate and keep in single cell suspension. We encountered much variation from experiment to experiment, and complete cell recoveries were not achieved. The use of complement-mediated lysis protocols to enumerate lymphocytes might be an additional source of error. Consequently, it is difficult for us to estimate the actual lymphocyte content of the different tumors. However, assuming the cell recovery is a stochastic event, and the cells recovered are a representative sample of the whole tumor, we can calculate the percentage of each cell type in a tumor. This calculation makes it apparent that tumors of subpopulation 168 contain many fewer lymphocytes than do tumors of the other four lines tested (Table 4). Tumor 168 is the most resistant of the lines to primary immune mechanisms, i.e., tumor growth in normal syngeneic mice is not very different from growth in immunosuppressed mice (Table 1). Line 168 tumors are unable to induce transplantation immunity in BALB/cfC_3H mice, although they are sensitive to such immunity induced by 410 tumors [10]. Thus, in this series of tumors, a low lymphocyte content correlates with weak immunogenicity.

In addition to total lymphocyte content, another clear difference between the tumor lines is the relative distribution of T lymphocyte subpopulations within the lymphocyte fractions isolated from the tumors. Tumor 68H infiltrates were enriched for Lyt 1.2^+ cells; the ratio of Lyt 1.2^+ : Lyt 2.2^+ cells was greater than 1. The Lyt 1.2^+ : Lyt 2.2^+ ratios for the other tumors were less than 1. This pattern perhaps is related to one of the properties by which 68H tumors differ from the other lines, namely, low tumorigenicity, as manifested by a long latency period and the necessity for high tumor cell inocula [5].

Mule et al. [11] found that Lyt 1^+ lymphocytes, which were isolated from small methylcholanthrene-induced tumors and expanded by growth in the presence of interleukin 2, were able to inhibit growth of tumor cells in vivo. Conversely, Lyt 2^+ lymphocytes, similarly obtained from large tumors, stimulated tumor growth in vivo. The unique T cell subpopulation ratio shown by 68H tumors, versus those of the other tumors studied, may result from a differential traffic of lymphocyte subpopulations either into or out of 68H tumors. Alternatively, the ratio may reflect tumor influence upon lymphocyte development. The determination of Lyt-antigen specificity appears to be a post-thymic event, but it is not known whether this determination is pre-programmed or subject to control by local factors [13, 14]. Unlike the other tumor lines tested, 68H behaves as a stem-cell line, giving rise to variant populations when grown in vivo [7]. Perhaps differentiation signals involved in production of mammary tumors cell variants also favor development of Lyt 1^+ expressing cells. The possibility that tumors can influence the differentiation of their associated lymphoid cells is under investigation.

Unlike Mule et al. [11], we did not find that the presence of Lyt 1^+ vs Lyt 2^+ cells correlated with tumor size. The tumor lines used here were subpopulations from a single tumor [5, 8]; thus the Lyt phenotypes of tumor-associated lymphocytes appear to be yet another factor influenced by tumor heterogeneity. Perhaps the shift in T cells noted by Mule et al. was not a function of tumor size per se, but rather a reflection of shifts in tumor cell subpopulations during the course of tumor growth.

The variants of tumor 410 (410, 410.4 4501) all were infiltrated by significantly more Lyt 2^+ than Lyt 1^+ cells, regardless of latency period, metastatic phenotype, or ability to induce transplantation resistance. Thus, the relative numbers of T cell subpopulations do not correlate with these important behavior characteristics, at least in our tumor system.

In this study we concentrated on the analysis of T lymphocyte subpopulations using monoclonal reagents to cells of the T helper (Lyt 1^+) and T killer-suppressor classes (Lyt 2^+). Our protocol did not allow us to distinguish Lyt $1^+/2^+$ cells. Since in no tumor was the percentage of Thy 1.2 cells significantly less than the combined percentage of Lyt 1^+ and Lyt 2^+ cells, there is a priori no reason to suspect the presence of high number of double-marked cells.

In previous work directed toward the functional characterization of tumor-associated lymphocytes, we did not detect cells capable of inhibiting tumor growth, in vivo or in vitro, in lymphocytes isolated from mammary tumors. Indeed, our experience has been that tumor-associated lymphocytes, including those from tumor 410, are stimulatory to tumor growth [3, 4]. Further studies are being carried out on the functional activity of lymphocytes from the other tumors studied here. Additional work in our laboratory is directed towards characterization of other types of host cells within tumors. Of particular interest is the observation that cytotoxic tumor-associated macrophages are found predominantly in tumors that can metastasize [9]. Macrophages are present, in equal numbers, in non-metastasizing tumors, but they are only weakly cytotoxic at best. These observations, and the present data, further emphasize the necessity of dissecting the role of host antitumor reactions in regard to specific facets of tumor behavior [6]. Our data at this point suggest that tumor-associated T cells may correlate with tumor growth, whereas intratumoral macrophage function is associated with metasta-

sis. If a cause-effect relationship exists, in both cases, the host component may be aiding the tumor. Further studies with tumors of defined behavior patterns may lead to an understanding of how malignant cells are able to co-opt the 'defense' reactions of the host.

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