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Purified mouse mammary tumor and lymphoid cells in immune assays

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Summary. *Tumor and lymphoid cell components from primary mammary adenocarcinomas of C3H/He mice were isolated simultaneously by velocity gradients. Viable tumor cells were obtained in sufficient numbers to test their in vivo and in vitro growth. Isolated tumor cells grew in 97% of inoculated syngeneic animals. In six assays with different tumors the effects of tumor-associated lymphoid cells (TAL) on in vivo tumor growth varied, enhancing in three and delaying in two experiments. Isolated tumor cells from animals with enhancing TAL grew faster in nonirradiated mice, whereas tumor cells from animals with inhibitory TAL grew better in irradiated animals. Isolated tumor cells also proliferated in cell culture, where they averaged 35% primary plating efficiency. Separated tumor cells were used in short-term ^{51}Cr-release assays with TAL, tumor-bearer lymph node and spleen effectors. Cytotoxicity was detected in only five of 25 assays. In no case was there killing by lymphocyte populations from normal animals. In the present report we describe a technique for the isolation of viable tumor and lymphoid cells from murine adenocarcinomas that allows study of interactions between these populations from the original tumor-bearing host.*

Introduction

Interest in the functional capability of lymphoid cells associated with neoplasms is a recent development in tumor immunology (for reviews see [10, 12]). Although TAL have been found to affect tumor growth in animal models, few experiments have used freshly separated tumor cells as targets. It has been shown that human tumor cells are sensitive to natural lymphocyte-mediated cytotoxicity only a few days after explantation, whereas they have little sensitivity when tested directly [11]. Tumor cell lines carried in vitro are lysed more readily in cytotoxic assays than are the same cells passaged in vivo [9]. It is important, then, to test the function of TAL on freshly isolated target cells before reaching general conclusions about their activity. In previous experiments [1] with strain BALB/cfC_3H spontaneous mammary tumors, lymphocytes were separated by isokinetic gradients [15]; this procedure yielded populations highly enriched in lymphoid cells but with insufficient numbers of freshly isolated tumor cells for experiments. In these earlier studies tumor cells were grown in culture for 7 days and then used as target cells. TAL had a stimulatory effect on tumor cell growth in vitro [3] and in vivo [4]. We report here a procedure by which cell suspensions from spontaneous C3H murine mammary tumors were separated to yield fractions enriched in tumor cells and fractions enriched in lymphoid cells. Isolated tumor cells grew well both in vivo and in vitro and were usable as targets in assays of cell-mediated cytoxity.

Materials and methods

Tumors. The tumors in this study arose spontaneously in female C3H/He mice. For evaluation of the in vivo growth potential of the separated cells, 1×10^5 viable tumor cells were injected SC into 2- to 4-month-old syngeneic mice. Viability was assessed by trypan blue exclusion. Some mice were irradiated (400 R) 1 h before tumor cell inoculation.

Lymph node and spleen cells. Lymph nodes and spleens were teased with needles to obtain suspensions. Erythrocytes were eliminated from the spleen suspensions by hypotonic lysis. The cells were washed twice in medium, counted, and adjusted to the appropriate concentration.

Separation of tumor and lymphoid cells. Tumor specimens passed through stainless steel mesh were mixed with serum-free medium containing collagenase (3 mg/ml) and DNAse (0.2 mg/ml). The suspensions were stirred with a magnet for 60 min and then washed once with medium. Approximately 5×10^7 cells in 10 ml medium were layered over FCS velocity gradients and sedimented for 1 h at room temperature (see Table 1, step 1). The cells in the sediment and the cells in the interphase were collected separately. The cells in the sediment were centrifuged, resuspended in fresh medium and resedimented on a second FCS gradient (step 2B). The cells were collected and checked for viability with trypan blue, and their composition was determined after exposure to Turk's solution, which stains nuclei of cells. A more thorough characterization of the cell morphologies was carried out later on May-Grunwald-Giemsa-stained smears. The cells at the interphase of step 1 were washed, resuspended in fresh medium, and layered over a second FCS gradient (step 2A). The cells at the interphase were collected, washed, and layered over 20 ml Ficoll-Isopaque (density 1.077) to a maximum of 50×10^6 (step 3). The tubes were centrifuged at

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Table 1. Protocol of cell separation for tumor no. 6 (weight 1.5 g, yield 1.7×10^7 viable tumor cells; 3.1×10^6 lymphoid cells)

Nucleated cells	% Composition of the suspensions	Step 1			Step 2A			Step 2B			Step 3		Step 4
		FCS velocity gradient			FCS velocity gradient			FCS velocity gradient			FI density gradient		Adherence on plastic
		Sediment	FCS	Interphase	Sediment	FCS	Interphase	Sediment	FCS	Interphase	Pellet	Interphase	
Viable tumor cells	25	83	80	8	85	90	5	93	72	10	3	8	3
Lymphoid cells	3	1	1	7	2	1	9	1	3	4	0	89	97
Macrophages	2	12	3	2	5	3	1	1	5	6	0	3	0
Other nontumor cells	5	2	14	2	5	4	1	1	5	10	0	0	0
		Pooled To step 2B		To step 2A	Discarded		To step 3	To experiment		Discarded	Discarded	To step 4	To experiment

800 *g* for 20 min. Cells were recovered from the interphase, suspended in fresh RPMI-1640 with 20% FCS, filtered through 20 μm Nytex to remove aggregates, and placed in large tissue culture flasks for adherence at 37° C for 18 h (step 4). The lymphocyte fractions were assessed for composition and viability after staining with Turk's solution and trypan blue.

Autologous lymphocytotoxicity (ALC)-test. Target cells (10^6) were labeled in 0.5 ml RPMI + 10% FCS by addition of 100 μCi sodium ^{51}Cr-chromate, specific activity 100–350 μCi/μg (Radiochemical Centre, Amersham). Following incubation for 2 h at 37° C they were washed four times and resuspended in RPMI + 10% FCS. The target cells (5×10^3/well) were dispensed into wells of round-bottom microplates and lymphocytes were added to give an effector-to-target ratio of 50 : 1. The final volume in each well was 0.2 ml. All tests were performed in triplicate. Following 4 h incubation at 37° C, 0.1 of the supernate was collected and the radioactivity in supernatant and remaining pellet was counted [17]. Spontaneous ^{51}Cr release was measured in samples of target cells incubated in medium and maximum ^{51}Cr release was determined by lysis of the cells with Triton X-100.

Percentage ^{51}Cr release from each tube was calculated from the formula:

$$\% \ ^{51}\text{Cr release} = \frac{2 \times \text{Counts in supernate}}{\text{Total counts in supernate and pellet}} \times 100 .$$

Cytotoxicity was derived from the formula:

$$\% \text{ Cytotoxicity} = \frac{\% \text{ Release in test} - \text{Spontaneous release}}{\text{Maximum release} - \text{Spontaneous release}} \times 100 .$$

Tests in which the spontaneous release exceeded 25% were discarded. Results were evaluated with reference to control lymphocytes by the Mann-Whitney U-test.

Results

Separation of cells

Primary C3H mammary tumors 8–12 mm in diameter were selected for study; smaller tumors had insufficient numbers of cells and larger tumors were usually necrotic. Histologically, TAL were seen at the periphery and rarely infiltrated the tumor mass. The percentage of TAL in the unprocessed suspension varied from tumor to tumor, ranging between 0.5% and 5% of the nucleated cells.

In the separation process (Fig. 1) tumor cell suspensions were layered over FCS gradients (step 1). TAL were purified from the cells collected at the interphase by applying to a second FCS gradient (step 2A); contaminating erythrocytes were removed on a Ficoll-Isopaque density gradient (step 3), and for some studies the remaining tumor cells were eliminated by adherence (step 4). Viable tumor cells were recovered in the sediment of the first FCS gradient and further purified on a second FCS gradient (step 2B). Many tumor cells, damaged in the process of preparing the cell suspension, were not recoverable. Separated tumor cells from step 2B were then used in experiments. The cellular composition of the fractions is exemplified in Table 1. Cell recoveries varied among the different experiments, ranges being 20%–50% of the tumor and 60%–80% of the lymphoid cells estimated in the initial suspension. TAL suspensions that contained more than 4% tumor cells were not used. Large cells with large nuclei were considered to be possible tumor cells for this evaluation.

Tumor cell suspension from step 2B were filtered through Nytex to remove aggregates and seeded into microplates at three concentrations. Fifteen different primary tumors were processed, and cells from them had an average primary plating efficiency of 35% (range 5%–55%). After 48 h the cells in the plates were epithelial in appearance, with large nuclei and prominent nucleoli (Fig. 2). These cultures were used in growth inhibition assays [1].

In vivo growth of isolated tumor cells

Separated tumor cells were tested for SC growth in syngeneic mice. The gradients were performed in duplicate (step 1): from half the gradients only the sedimenting tumor cells (pellet step 2B) were collected, whereas from the remaining gradients both TAL (interface step 3) and tumor cells were collected. Tumor cell suspensions were immediately inoculated into syngeneic mice both with and without TAL (2×10^5 lymphoid cells). Isolated cells from all the separated tumors grew. In a series of six experiments with different primary tumors the effects of TAL on the in vivo growth rate varied with the tumor tested. In three experiments the presence of TAL enhanced and in two experiments TAL delayed the growth of cells. In one

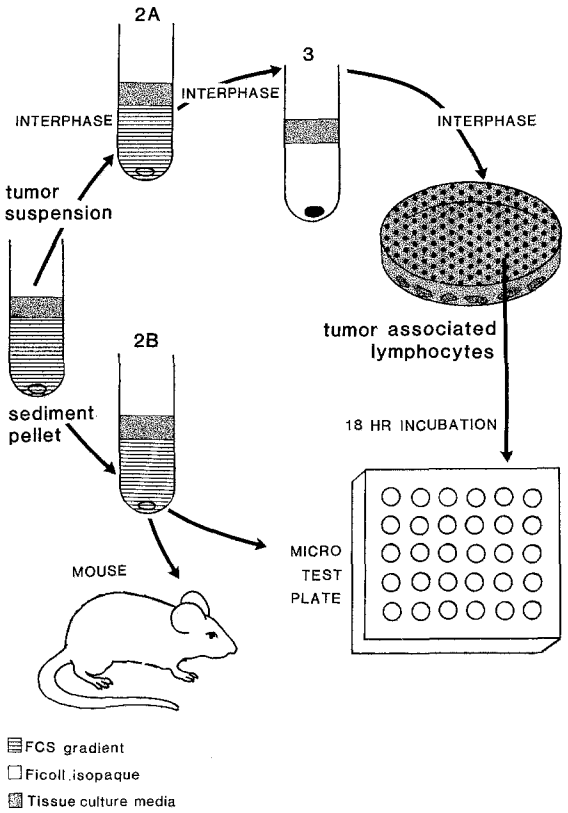

Fig. 1. Separation of tumor and lymphoid cells. Tumor cell suspensions were layered over FCS gradients. Sedimenting cells were collected, resuspended, and applied to a second FCS gradient (step 2B); this sediment was collected and used in experiments. Lymphoid cells were collected in the interphase of the first gradient, resuspended and applied to a second FCS gradient (step 2A); cells at the interphase of this second gradient were collected, resuspended, and layered over a Ficoll-Isopaque gradient (step 3); cells at the interphase were collected and resuspended in tissue culture flasks for 18 h; TAL were collected as unattached cells from the flasks and used in experiments

experiment there was no difference between the growth of the tumor suspensions with or without TAL. Results of two representative experiments with TAL from two different primary tumors that had opposite effects are presented in Table 2. The TAL suspensions used for this study contained some tumor cells (range 3%–10%). The percent tumor cell contamination did not correlate with in vivo tumor growth.

In vivo growth of isolated tumor cells in irradiated mice

Doses of 1×10^5 tumor cells were implanted SC into irradiated and/or normal mice. Some tumors grew faster in irradiated mice, whereas others grew faster in normal mice. These results resembled the findings for tumor cell growth with and without TAL discussed above. In Table 2 results of tumor growth in irradiated mice are presented along with the data on TAL

Table 2. In vivo growth of isolated tumor cells

	TAL added[a]	Irradiated recipients[b]	Tumor incidence[c]	Latent period[d]	Growth rate[e]
Tumor A	–	+	3/4	23 ± 3	18 ± 2*
	+	+	3/3	21 ± 1	27 ± 2*
	–	–	5/5	22 ± 3	43 ± 1
Tumor B	–	+	5/5	29 ± 2	32 ± 2*
	+	+	5/5	36 ± 1	20 ± 1*
	–	–	1/5	35 ± 1	9 ± 3

[a] 2×10^5 TAL added to 1×10^5 tumor cells
[b] 400 R was given 1 h prior to tumor injection
[c] Tumor incidence is expressed as number of animals in which tumor grew/total no. of animals that received injections of tumor cells
[d] The mean latent period ± SE
[e] Mean slope and SE for growth (mm^2/day)
* $P < 0.01$ (Mann-Whitney nonparametric test)

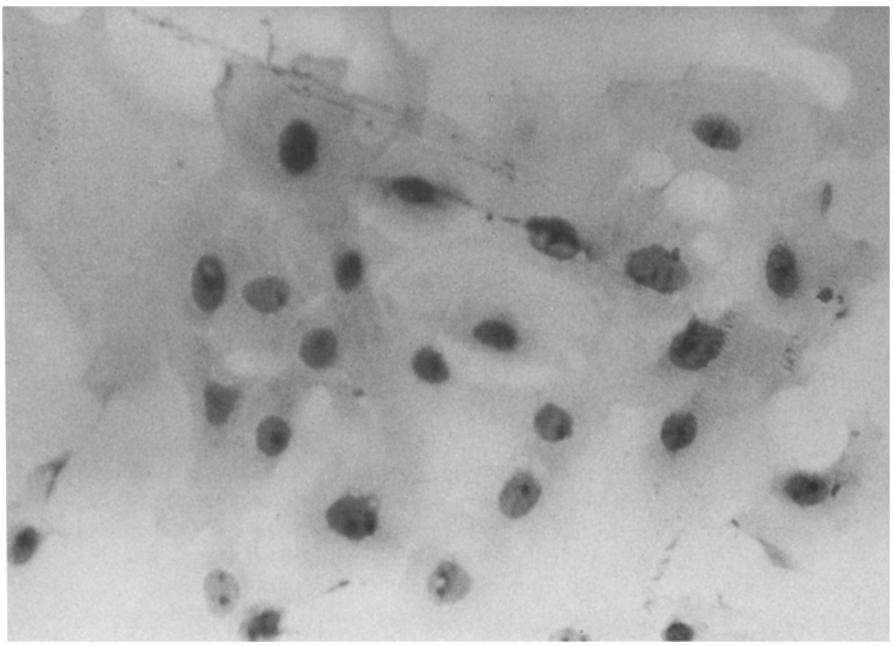

Fig. 2. C3H mammary tumor cells. May-Grunwald-Giemsa stain of separated mammary tumor cells in primary culture. 350×

Table 3. ^{51}Cr release assay with isolated tumor cell targets

Tumor	% Specific ^{51}Cr release with effectors[a] from				
	Tumor bearer			Control	
	TAL	LN	S	LN	S
1	2	3	7	0	3
2	0	25*	4	2	4
3	–	7	10	3	3
4	–	16*	5	4	1
5	–	9	12	0	3
6	–	28*	0	4	2
7	21*	1	3	1	2
8	–	9	0	3	0
9	10*	0	0	0	3
10	5	9	6	2	0

[a] 50 : 1 effector-target ratio
* $P < 0.05$ compared with control cells

effects. Lymphoid cells augmented the growth of tumor A: this tumor grew faster in nonirradiated mice and the addition of TAL to the inoculum enhanced the growth rate. In contrast, lymphoid cells inhibited the growth of tumor B: these cells grew better in irradiated animals and the admixture of TAL to the inoculum reduced the growth rate.

Isolated tumor cells as cytotoxic targets assayed by ^{51}Cr release

^{51}Cr release values from 10 experiments with different tumors exposed to various lymphoid cell population are summarized in Table 3. Although assays were conducted at effector-to-target ratios of both 50 : 1 and 20 : 1, little activity was observed with the latter, so only the results with the higher ratio are shown. Tumor-bearer lymph node cells (LN) were cytotoxic in three of the 10 separate experiments (tumors 2, 4, and 6). Spleen (S) cells from these animals were not cytotoxic. TAL were obtained in sufficient numbers for testing in five of the 10 experiments, and in two experiments they were cytotoxic (tumors 7 and 9). In one experiment the animal had two primary tumors (tumors 1 and 2). These were tested separately. The autologous LN were reactive against only one of these tumors, while neither the spleen cells nor the TAL were reactive. Initially TAL were used following step 3. Little antitumor reactivity was observed. The possibility existed, however, that the contaminating tumor cells might compete for lymphoid cell activity and reduce the level of detectable cytotoxicity. Therefore, we chose to use the more highly purified lymphoid cells without contaminating tumor cells. In this way, adherent lymphoid cells were eliminated from the TAL.

Discussion

We have separated primary strain C3H mouse mammary tumors into populations enriched for tumor and for lymphoid cells by velocity gradient separation. The isolated tumor cells grew SC in syngeneic mice. The removal of TAL from a series of different primary tumors had differing effects on the growth of these tumors. All three possible effects on tumor growth, i.e., enhancement, suppression, no effects, were seen following TAL removal from different tumor cell populations. However, with all these tumors, the influence of TAL on tumor growth paralleled the effects on tumor growth induced by immunosuppression in the form of whole-body irradiation of recipients before tumor cell inoculation. This suggests that in situ immunity as measured by the influence of TAL on tumor growth in vivo foreshadows the character of the immune response seen with grafts from a tumor. These data expand our earlier studies with lymphoid cells from one murine tumor line with which growth stimulation was observed [4]. Previous studies have shown that the relative distribution of the subpopulations comprising the lymphoid infiltrate was found to vary among a series of primary tumors [2]. Furthermore, murine mammary tumor sublines with differing biological properties vary in the amount of infiltrate that they attract and in the relative distribution of T lymphocyte subpopulations in the infiltrate [16]. This variability, which is reproducible, appears to be characteristic of the tumor rather than the host. Our present data suggest that the nature of the TAL cell populations correlate with tumor growth in vivo.

Separated cells were used as primary targets for cytotoxicity recorded by ^{51}Cr release. In short-term ^{51}Cr release assays lymphocyte-mediated cytotoxicity by TAL against the separated tumor cells was seen in only two of five experiments. There was no correlation between the effects of TAL and lymph node or spleen cell populations in the assays. This discrepancy among lymphoid cell populations is similar to our earlier findings with direct enumeration assays [3] and those of other laboratories as reviewed by Haskill [10]. In these studies, control lymphocytes did not lyse isolated tumor cell targets. Thus no NK effect was observed in these experiments.

Gradient separation makes it possible to carry out experiments with fresh tumor and lymphoid cells obtained directly from solid growths. Clearly the separation process itself imposes selectivity on the lymphoid cells recovered. Monocytes were essentially removed from TAL populations that were adhered. Monocyte-like cells comprised 3% (range 2%–6%) of the lymphoid cell population (step 3) before adherence. Unadhered lymphoid cells were used to performed in vivo growth experiments and for the initial ^{51}Cr-release assays. However, this method provides the investigator with tumor cells that are not selected by in vitro propagation for a particular cell type. Individual neoplastic cells within tumors are heterogeneous for several parameters such as cellular morphology [7], immunogenicity [14], karyotype [13], and metastatic ability [8]. For mouse mammary tumors heterogeneity has recently been demonstrated by sublines derived from one tumor which vary in many features, including in vivo and in vitro growth rates [6]. Cultured cells may not be representative for complete tumor cell populations. This separation procedure enables experiments to be performed to evaluate cellular composition and immune activity with tumor and lymphoid cells from the original host.

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