Separation of tumor-infiltrating lymphocytes from tumor cells in human solid tumors

A comparison between velocity sedimentation and discontinuous density gradients

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The separation of viable tumor-infiltrating lymphocytes (TIL) from surgical biopsies of human solid tumors was achieved by velocity sedimentation at unit gravity or by discontinuous density gradients. The two methods were adapted to small volumes and cell numbers not exceeding $1 \times 10^8$. The recovery, purity and composition of the TIL-enriched fractions were comparable in the two methods. Density gradients were more rapid, simpler and more practical for preparation under sterile conditions of TIL from clinical material than velocity sedimentation. Lymphocytes in the TIL-enriched fractions obtained by either of the methods were poorly responsive to mitogens. This poor responsiveness is a characteristic of the human TIL and seems to be related to effects exerted by tumor cells.

Key words: Separation of tumor cells and lymphocytes; Velocity sedimentation; Discontinuous density gradients; Human tumor-infiltrating lymphocytes

Introduction

In addition to tumor and tissue cells, many tumors contain infiltrating, host-derived populations: lymphocytes, macrophages, and granulocytes (Ioachim, 1979). A variety of cell separation methods have been used for isolation and characterization of tumor-infiltrating lymphocytes (TIL; for review see Pretlow and Pretlow, 1983). Most of these methods were applied to disaggregated animal tumors, where large quantities of cells could be obtained.

Velocity sedimentation at unit gravity, first described by Mel (1964), then modified by Peterson and Evans (1967) and by Miller and Phillips (1969), separates cells primarily on the basis of cell size and is a recommended procedure for separation of TIL in the animal model systems. Several effective modifications of this procedure have been described (e.g., MacDonald and Miller, 1970; Bont et al., 1979; Nethanel et al., 1981; Opstelten et al., 1982; Wells, 1983). The resolution obtained in this method depends on cell load and thickness of cell layer, viscosity of the sample, and size and dimensions of the sedimentation chamber (Bont and De Vries, 1977). The resolution can be somewhat improved and time shortened by use of the tilting
procedure devised by Bont et al. (1979). Also, centrifugal elutriation (Mackler et al., 1977; Meistrich, 1977) significantly increases separation capacity of velocity sedimentation, but it requires both large cell numbers and an expensive elutriator rotor. In mouse tumors, centrifugal elutriation was successfully used for the separation of sub-populations of normal and neoplastic cells (e.g., Meistrich et al., 1981; Siemann et al., 1981).

Differences in density as well as differences in size have been employed for separations of different types of cells, and several types of gradients suitable for such separations have been described (see Pretlow and Pretlow, 1983). For separation of TIL from tumor cells in human solid tumors, it is essential to have a method that is rapid, easy and adaptable to relatively small numbers of cells recovered from surgical biopsies. At the same time, the method of choice should achieve satisfactory separation, yield cells that are viable and functionally intact, give good recovery and avoid selective losses of cell populations. As individual human tumors are heterogeneous in terms of both cell size and cell density (e.g., Mackillop and Buick, 1981; Cillo et al., 1984), it is difficult to predict their behavior during a separation procedure.

We have attempted to separate TIL from over 40 human solid tumors and found that velocity sedimentation, although efficient in yielding homogeneous preparations of large tumor cells, was not superior to discontinuous density gradients in obtaining TIL-enriched preparations. Centrifugation on discontinuous density gradients was effective, less time-consuming and certainly more practical for separation of TIL from tumor and tissue cells. Functional studies performed with enriched TIL preparations indicated that these cells have a reduced ability to respond in mitogen-induced proliferative assays.

Materials and methods

Tumor tissues

Biopsies of human solid tumors were obtained from patients who had surgery for therapeutic reasons. Tissues were collected into sterile culture medium (RPMI 1640, Seromed, Basel, Switzerland) supplemented with 200 mM glutamine, antibiotics and mycostatin (all from Gibco, Basel, Switzerland) and processed immediately. All procedures and cell manipulations were done under sterile conditions. Disaggregation of tumor tissue by combined mechanical and enzymatic means was described by us earlier (Whiteside et al., 1986). Briefly, tissues were sliced in a Stadie-Riggs microtome (Stadie and Riggs, 1944), minced with razor blades, and then treated with an enzyme cocktail containing collagenase (0.05% w/v, CLS II, Worthington, Freehold, NJ) and DNase (0.002% w/v, DNase I, Sigma, Geneva, Switzerland) at 37°C with stirring for 2–18 h until complete dissolution of tumor tissue was achieved. Cell suspensions were filtered through nylon mesh screens (Nybolt, Société Suisse de Tissage de la Soie, Zurich, Switzerland) with pores of 50 and 25 μm to remove cell clumps. After extensive washing and additional filtrations, the tumor-derived cell mixtures were separated by velocity sedimentation or by density centrifugation as described below.

Cell separation by velocity sedimentation

Cells were resuspended in 2 ml of 5% (v/v) fetal calf serum (FCS, Seromed, Basel) in growth medium. Before separation, cell viability was determined and the concentration adjusted to not more than 6 × 10^6/ml. A modified procedure of Miller and Phillips (1969) scaled down to accommodate low cell numbers in a small volume was used. Cell suspensions were layered on a buffered linear gradient of 15–30% FCS in medium (total volume 42–44 ml) at 4°C in a closed cylindrical glass chamber (diameter, 3 cm; height, 16 cm). Cells were allowed to sediment at unit gravity for 3 h at 4°C. Fractions of 1 ml were collected, concentrated to 0.2 ml by centrifugation and assayed for cell numbers and cellular morphology. Sedimentation velocity was arbitrarily expressed in mm/h.

The separation method was first used with mixtures of normal peripheral blood mononuclear cells (MNC) and various tumor cell lines maintained in culture. Mixtures of tumor cells labeled with ^51Cr (IRE, Belgium, spec. act. 5 mCi/ml) or ^3H]leucine (Amersham, Zurich, spec. act. 5 mCi/ml) and of unlabeled MNC (or labeled MNC and unlabeled tumor cells) were used to determine
optimal conditions for separation, gradient capacities, and relative sedimentation velocities for cells of different sizes. In addition, MNC and blasts generated in a 6 day mixed lymphocyte culture were fractionated. Following the separation, cell fractions were assayed for (a) radioactivity; (b) cell numbers and viability; (c) morphology.

Tumor-derived cell suspensions from three metastatic melanomas in which tumor cells were several-fold larger than TIL were chosen for separation by velocity sedimentation. In addition, cells obtained from the blood of a patient with a biphenotypic leukemia (monocytic/lymphoblastic) were separated. Peripheral blood lymphocytes used in velocity sedimentation experiments were obtained from random normal donors at the blood bank.

**Cell separation by discontinuous density gradients**

The TIL were separated from tumor and tissue cells on the basis of density by centrifugation on discontinuous Ficoll-Hypaque gradients as described by Vose (1982). Suspensions of disaggregated tumors (generally in 5 ml of medium) were placed on top of the gradient formed by overlaying a cushion of 100% Ficoll-Hypaque (density 1.077, Pharmacia, Uppsala, Sweden) with an equal volume of 75% (v/v) Ficoll-Hypaque in RPMI 1640 (density, 1.055). Gradients (25 ml) were centrifuged at 800 × g for 20 min at room temperature. The MNC-enriched fractions which formed a distinct band at the interface between 75% and 100% Ficoll-Hypaque were collected, washed twice in fresh medium and counted in a hemacytometer. Tumor cells, which formed the upper band on top of 75% Ficoll-Hypaque were also collected.

Suspensions of the following 40 solid tumors were separated on discontinuous density gradients: 6 melanomas, 14 breast, 3 oesophageal, 2 lung, 5 colon, 1 basal-cell, 3 ovarian carcinomas and 6 gliomas. Characteristics of these suspensions as well as histopathologic diagnoses of the tumors were described in detail elsewhere (Whiteside et al., 1986).

**Cell counts and staining**

The numbers of viable TIL and tumor/tissue cells were determined by counting samples in a hemacytometer in the presence of trypan blue. Morphology and differential cell counts were done on cytopsin preparations which were air dried, fixed and stained with May-Grünwald-Giemsa (MGG). These counts were performed independently by two observers, and on each slide, 150–200 cells in consecutive fields were counted.

Numbers of T lymphocytes in the final lymphocyte-enriched preparations were determined by immunofluorescence using a microtechnique described earlier (Whiteside et al., 1986). Monoclonal pan-T antibody T11 was from Coulter Electronics, Geneva, Switzerland. Two-color fluorescence was performed to determine the presence of receptors for IL-2 and HLA-DR antigens on T lymphocytes isolated from tumors. Phycoerythrin- and fluorescein-labeled monoclonal antibodies to human IL-2 receptors and HLA-DR antigens were purchased from Becton Dickinson, Basel, Switzerland. Prior to immunofluorescence, all cell suspensions were incubated in medium at 37°C in 5% CO₂-air atmosphere for up to 18 h to allow for re-expression of surface receptors that might have been removed during tissue disaggregation with enzymes. In control experiments performed with tonsils that were disaggregated with enzymes exactly like tumor suspensions, such an incubation was necessary for normal re-expression of lymphocyte surface antigens.

**Lymphocyte cultures and thymidine uptake**

After separation, cell fractions containing lymphocytes were pooled and washed twice in medium. Lymphocytes were plated on the basis of the T11⁺ cell numbers in 96-well round-bottom microtiter plates (no. 3799, Costar, Cambridge, MA). Each well contained 5 × 10⁴ T lymphocytes in 100 μl of culture medium supplemented with 10% FCS. In some experiments, irradiated autologous mononuclear cells were added (2.5 × 10⁴/well) as a source of adherent cells. Phytohemagglutinin (PHA, 1% v/v, final concentration, Gibco) was added in 100 μl of medium/well. Cultures were incubated for 48 h at 37°C in 5% CO₂-air atmosphere. [³H]thymidine (Amersham, U.K., specific activity 6 Ci/mmol) was added (1 μCi/well) for the last 12 h of culture. Cells were harvested using a multiple automated harvester (Dynatech), and the incorporation of [³H]thymi-
dine in triplicate cultures determined by standard
techniques of liquid scintillation spectroscopy.

**Mixed lymphocyte cultures**

Peripheral blood lymphocytes (1 × 10^6/ml) were incubated with 1 × 10^6/ml irradiated (5000 rads.) allogeneic peripheral blood lymphocytes in 24-well Costar plates for 6 days at 37°C in 5% CO_2-air atmosphere. [3H]thymidine incorporation was measured in one set of triplicate wells pulsed 12 h before harvest. The remaining cells were harvested, washed 3 times and the numbers of blasts vs. small lymphocytes determined on MGG smears. The blasts were separated from small lymphocytes by velocity sedimentation.

**Limiting dilution assay**

Peripheral blood lymphocytes, TIL or MLC blasts were diluted in medium and seeded in limiting numbers in round-bottom wells of 96-well Costar plates containing 5 × 10^4 irradiated (5000 rads.) allogeneic spleen cells under conditions described by Moretta et al. (1983). PHA (Gibco, 1%, v/v) was added at the onset of culture. After 48 h, IL-2-containing supernatants (50% v/v) prepared exactly as described by Moretta et al. (1981) were added. Cultures were supplemented weekly with irradiated feeder cells suspended in 100 µl of IL-2-containing supernatant. Plates were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. After 14–25 days in culture each microwell was microscopically assessed for growth. Minimal estimates of the proliferating-lymphocyte precursors were obtained by the χ^2 method from the Poisson distribution relationship between the responding cell number and the logarithm of the percentage of non-responding (negative) microcultures (Taswell, 1981).

**Tumor cell lines**

Tumor cells were maintained in medium containing 10% FCS. Adherent tumor lines were passaged using trypsin and EDTA. Melanoma lines IGR3 and Me8 as well as Jurkat, a mature T-cell leukemia line, and the human erythroleukemia line K562, were obtained from Dr. S. Carrel, Ludwig Institute, Epalinges. The human breast line MCF7 was from Dr. H. Diggelmann, ISREC, Epalinges.

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### Results

**Velocity sedimentation of tumor-PBL mixtures**

In order to establish the conditions optimal for separation of tumor-infiltrating lymphocytes (TIL) by velocity sedimentation at unit gravity, we applied this separation procedure first to normal peripheral blood lymphocytes (PBL) and then to mixtures of tumor cells and PBL. Fig. 1 shows a typical separation profile for a mixture consisting of 3 × 10^6 melanoma cells (line IGR3) and 10 × 10^6 PBL. The tumor cells were labeled with [3H]leucine and the PBL with ^51^Cr prior to the separation procedure. The separation between the two types of cells was excellent; the recovery of the tumor cells approached 75% and of the PBL 95% as determined by cell counts; morphologic examination of the MGG smears made of the peak fractions confirmed their purity (see Fig. 2); and the separation was achieved in 3 h. The heterogeneity of tumor cells with respect to size is apparent in Fig. 1 (from 18 to 35 µm). In contrast, PBL always gave a well-defined narrow peak at the sedimentation velocity of 4.9 ± 0.5 mm/h (Ta-

### TABLE I

VELOCITY SEDIMENTATION AT UNIT GRAVITY OF NORMAL HUMAN PERIPHERAL BLOOD LYMPHOCYTES, MLC BLASTS AND DIFFERENT TUMOR CELLS MAINTAINED IN CULTURE

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Cell diameter a (mean µm ± SD)</th>
<th>no. of experiments</th>
<th>Sedimentation velocity (mean mm/h ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBL</td>
<td>10.4 ± 1.6</td>
<td>10</td>
<td>4.9 ± 0.5</td>
</tr>
<tr>
<td>PBL-T b</td>
<td>9.5 ± 1.6</td>
<td>1</td>
<td>4.8</td>
</tr>
<tr>
<td>MLC blasts</td>
<td>16.0 ± 2.1</td>
<td>2</td>
<td>10.8 ± 0.2</td>
</tr>
<tr>
<td>Jurkat</td>
<td>16.3 ± 2.7</td>
<td>2</td>
<td>9.7 ± 0.5</td>
</tr>
<tr>
<td>K562</td>
<td>20.5 ± 2.6</td>
<td>4</td>
<td>13.7 ± 0.9</td>
</tr>
<tr>
<td>Me8</td>
<td>24.3 ± 3.6</td>
<td>3</td>
<td>14.2 ± 1.8</td>
</tr>
<tr>
<td>IGR3</td>
<td>26.5 ± 3.9</td>
<td>3</td>
<td>17.3 ± 0.4</td>
</tr>
<tr>
<td>MCF7</td>
<td>29.0 ± 4.9</td>
<td>2</td>
<td>18.7 ± 1.0</td>
</tr>
</tbody>
</table>

a Diameters of cells in cytocentrifuge preparations stained with MGG were measured using a light microscope with a graticule inserted into a ×10 eye piece (obj. ×100). 50 cells were measured and mean sizes ± SD were calculated for each cell type.

b PBL-T were obtained by passing normal peripheral blood lymphocytes through a nylon wool column.
Fig. 1. Separation by velocity sedimentation of human melanoma cells (line IGR3) and normal peripheral blood lymphocytes. The two populations were mixed together, and the mixture (containing $3 \times 10^6$ melanoma cells and $10 \times 10^6$ PBL) was separated at unit gravity on a 15–30% linear gradient of fetal calf serum. IGR3 cells were prelabeled with $[^3]$H]leucine (•), while PBL were labeled with $[^5]$Cr (○). Following the separation, cell numbers (□) were determined microscopically in fractions containing tumor cells and PBL. The mean sedimentation velocity calculated for IGR3 cells was 26.6 mm/h and for PBL 4.9 mm/h.

Table I. Equally impressive was the purity achieved in both the tumor and PBL fractions (see Fig. 2). The gradient capacity was defined, and from $3 \times 10^6$ to $15 \times 10^6$ cells could be successfully separated in our system. There was no evidence of streaming at these cell concentrations. This kind of separation was consistently seen with in vitro prepared mixtures of PBL and various tumor cells. The method was highly reproducible, and we were able to calculate sedimentation velocity values for cells with different sizes (diameters) as shown in Table I.

Velocity sedimentation of patient samples

Suspensions prepared by enzymatic digestion of three human melanomas were separated by velocity sedimentation at unit gravity. These suspensions were chosen because of large differences in size between tumor cells and mononuclear cells as determined by microscopic observations. In each of the three suspensions, the TIL represented a small component (2–5%) of total cells present. Before layering these suspensions on gradients, it was essential to remove clumped cells by repeated filtrations through nylon mesh screens with 50 and 25 μm pore diameter. The following numbers of cells were placed on the gradients: tumor 1 = $1.5 \times 10^6$; tumor 2 = $12 \times 10^6$; tumor 3 = $8.5 \times 10^6$. Separations, performed under the conditions established with tumor cell-PBL mixtures, yielded TIL-enriched fractions which contained between 30 and 56% of lymphocytes (Table II). The recovery of TIL, calculated on the basis of MGG smears before and after the separation, ranged from 60 to 100%. The viability of the recovered cells was > 95%. Fig. 3 shows the velocity sedimentation distribution of tumor cells and TIL obtained from a human melanoma. The TIL peak was broader than that in Fig. 1 for PBL and the sedimentation velocity somewhat higher for the
TIL (Table II), even though the TIL were not larger than PBL as determined by measurements of cell diameters on MGG smears. The tumor-cell fraction was again heterogeneous in terms of cell size, and it contained an almost pure population of viable tumor cells (Fig. 4; Table II).

Using a sedimentation velocity gradient, we also separated lymphoblasts from monocytic blasts in one case of biphenotypic leukemia (Table II). While the recovery of both monoblasts and lymphoblasts was > 95%, the enrichment in lymphoblasts compared unfavorably to that in monoblasts (45% vs. 94%, Table II).

These experiments indicated that, in contrast to in vitro prepared mixtures of cells, suspensions obtained from pathologic specimens were difficult to separate. In general, large cells (e.g., tumor cells, monoblasts) were recovered as nearly pure populations, while the TIL, although enriched, did not achieve the same degree of purity.

Separation of TIL on density gradients

We separated the TIL from over 40 human solid tumors using discontinuous density gradients (Whiteside et al., 1986). Both the recovery and purity of isolated TIL fractions varied widely from tumor to tumor. In several cases, we obtained preparations of TIL that were nearly free of contaminating tumor/tissue cells (see Fig. 5). More often, however, these fractions contained from 40 to 60% of lymphoid (T11+) cells. As an example, cellular compositions of the TIL-enriched fractions isolated from three human melanomas on density gradients are listed in Table III. Similar
TABLE II
VELOCITY SEDIMENTATION OF HUMAN TUMOR-INFILTRATING LYMPHOCYTES (TIL), BLASTS FROM A BIPHENOTYPIC MONOCYTIC/LYMPHOBLASTIC LEUKEMIA AND TUMOR CELLS ISOLATED FROM PATIENTS WITH MALIGNANCIES

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Cell diameter (mean μm ± SD)</th>
<th>Velocity sedimentation (mm/h)</th>
<th>Recovery %</th>
<th>Purity %</th>
</tr>
</thead>
<tbody>
<tr>
<td>TIL (1)</td>
<td>9.9 ± 1.3</td>
<td>5.5 (7.0–4.1) b</td>
<td>62</td>
<td>50</td>
</tr>
<tr>
<td>(2)</td>
<td>10.2 ± 1.6</td>
<td>6.5 (7.5–4.0)</td>
<td>60</td>
<td>30</td>
</tr>
<tr>
<td>(3)</td>
<td>9.7 ± 1.4</td>
<td>4.9 (6.3–3.0)</td>
<td>100</td>
<td>56</td>
</tr>
<tr>
<td>Tumor cells (melanoma) (1)</td>
<td>20.3 ± 2.2</td>
<td>11.0 (15–10)</td>
<td>30</td>
<td>100</td>
</tr>
<tr>
<td>(2)</td>
<td>21.6 ± 3.5</td>
<td>12.0 (17–7.8)</td>
<td>60</td>
<td>100</td>
</tr>
<tr>
<td>(3)</td>
<td>23.0 ± 3.6</td>
<td>15.0 (23–7.7)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Monocytic blasts</td>
<td>18.0 ± 2.2</td>
<td>9.4 (11.5–7.0)</td>
<td>97</td>
<td>94</td>
</tr>
<tr>
<td>Lymphoblasts</td>
<td>13.0 ± 1.2</td>
<td>6.0 (7.8–4.4)</td>
<td>97</td>
<td>45</td>
</tr>
</tbody>
</table>

a Purity of TIL preparations obtained from three malignant melanomas was estimated by counting lymphocytes on MGG smears and by determining percent of TIL+ lymphocytes/fraction by immunofluorescence. Purity of tumor cell preparations was determined on MGG smears of the separated cell fractions.
b Ranges are given in parentheses.

Fig. 3. Separation by velocity sedimentation at unit gravity of tumor-infiltrating lymphocytes (TIL; ○——○) and melanoma cells (○–○) from an enzymatic digest of human malignant melanoma. The cells (1.5×10^6 in 2 ml of 5% FCS) were separated on the gradient under conditions detailed in Fig. 1. Cell counts in trypan blue and morphological analysis were performed on each fraction to determine the viability, number, and morphology of recovered cells.

data were obtained with many other TIL populations isolated from different tumors (Whiteside et al., 1986). Non-hematopoietic cells were the main contaminant, whereas granulocytes or macrophages represented a minority of cells in these fractions. As shown in Table III, there was no difference in the cellular content of the TIL-enriched preparations obtained from six melanomas, three separated on velocity sedimentation and the other three on density gradients. The tumor cell fractions obtained on discontinuous density gradients had a high degree of purity (Fig. 5), but a quantitative recovery was often low (Table IV). As we were mainly interested in the TIL-enriched fractions, the loss of a substantial number of tumor cells was not a disadvantage. The enrichment in the TIL on discontinuous density gradients was consistently in the order of 30–40-fold. These gradients were easy to perform, economic in terms of time and reagents, and did not require special equipment.

Proliferative responses of lymphocytes following separation on gradients

To assure that lymphoid cells separated by velocity sedimentation were functionally intact, [3H]thymidine incorporation was measured in microcultures of cells incubated with PHA before
Fig. 4. Cell fractions obtained after separation by velocity sedimentation at unit gravity of an enzymatic digest of human malignant melanoma. A, a tumor-cell fraction (no. 25); and B, a fraction enriched in TIL (no. 36 in Fig. 3). May-Grünwald-Giemsa stain. Magnification × 530.

and after gradients. As can be seen in Table V, responses to PHA of normal PBL tested before and after velocity sedimentation were normal. Likewise, normal PBL showed unimpaired responses to PHA after density gradients (data not shown). However, normal PBL lost their ability to respond to PHA following mixing with tumor cells and separation on the gradient (Table V). Thus contact with tumor cells during a 3 h separation at 4°C was sufficient to inhibit PHA responsiveness of normal PBL.

Table VI shows that the TIL preparations obtained from several tumors and separated either by sedimentation velocity or discontinuous density gradients were also essentially unresponsive to PHA. As controls, we tested PHA responses of normal PBL and tonsil lymphocytes that were treated with the enzyme cocktail under the same conditions as tumor tissues and run on density
TABLE III
CELLULAR COMPOSITION OF THE TIL-ENRICHED FRACTIONS ISOLATED FROM SIX HUMAN MELANOMAS AND SEPARATED ON DIFFERENT GRADIENTS a

<table>
<thead>
<tr>
<th></th>
<th>Velocity sedimentation gradient n = 3</th>
<th>Density gradient n = 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocytes</td>
<td>45 ± 15 (34–56)</td>
<td>43 ± 2 (40–44)</td>
</tr>
<tr>
<td>Monocytes/macrophages</td>
<td>2 ± 0.4 (0–4)</td>
<td>1 ± 1.7 (0–3)</td>
</tr>
<tr>
<td>Granulocytes</td>
<td>4 ± 1 (3–5)</td>
<td>2 ± 1.5 (0–3)</td>
</tr>
<tr>
<td>Non-hematopoietic cells b</td>
<td>50 ± 12 (42–58)</td>
<td>50 ± 9 (40–56)</td>
</tr>
</tbody>
</table>

a Determined by morphology on W/G or MGG smears. 150–200 cells/smear were counted. Data presented as mean percentages ± SD. Ranges are given in parentheses.
b Tumor cells, tissue cells and cells that could not be identified.

gradients exactly like the TIL. The controls showed normal proliferative responses to PHA.

Limiting dilution analysis of lymphocytes separated on gradients
Normal PBL activated in a 7 day mixed

TABLE IV
TUMOR CELLS RECOVERED FROM ENZYMATIC DIGESTS OF HUMAN SOLID TUMORS FOLLOWING SEPARATION BY DISCONTINUOUS DENSITY GRADIENTS a

<table>
<thead>
<tr>
<th>Enzyme digest</th>
<th>Tumor cell enriched fraction</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>before gradient b</td>
<td></td>
</tr>
<tr>
<td>Melanoma</td>
<td>(1) 1 × 10^6</td>
<td>2.5 × 10^3</td>
</tr>
<tr>
<td></td>
<td>(2) 6 × 10^6</td>
<td>1.0 × 10^6</td>
</tr>
<tr>
<td></td>
<td>(3) 3 × 10^7</td>
<td>6.0 × 10^6</td>
</tr>
<tr>
<td>Breast CA</td>
<td>(1) 1 × 10^7</td>
<td>8.3 × 10^6</td>
</tr>
<tr>
<td></td>
<td>(2) 6 × 10^6</td>
<td>3.0 × 10^5</td>
</tr>
<tr>
<td></td>
<td>(3) 9 × 10^6</td>
<td>6.0 × 10^5</td>
</tr>
<tr>
<td></td>
<td>(4) 3 × 10^6</td>
<td>3.6 × 10^5</td>
</tr>
<tr>
<td>Oesophageal CA</td>
<td>(1) 7 × 10^6</td>
<td>1.0 × 10^6</td>
</tr>
<tr>
<td></td>
<td>(2) 4 × 10^6</td>
<td>1.5 × 10^6</td>
</tr>
</tbody>
</table>

a Tumors were disaggregated by a combination of mechanical and enzymatic methods, and the enzymatic digests were centrifuged, washed and separated on density gradients. Cell counts were made on MGG smears of unseparated enzymatic digests and tumor-enriched fractions obtained from gradients.
b For the purpose of calculating recovery of tumor cells, we assumed that the unseparated enzymatic digests contained 90% of tumor cells.

TABLE V
RESPONSES OF LYMPHOCYTES TO PHYTOHEMAGGLUTININ (PHA) FOLLOWING CONTACT WITH TUMOR CELLS DURING VELOCITY SEDIMENTATION GRADIENTS (SVG) a

<table>
<thead>
<tr>
<th></th>
<th>[3H]thymidine incorporation cpm × 10^-3 ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before SVG</td>
</tr>
<tr>
<td></td>
<td>+ PHA (SI)</td>
</tr>
<tr>
<td>n-PBL</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>n-PBL-T + Me8 b</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>n-PBL + Me8</td>
<td>1.5 ± 0.5</td>
</tr>
<tr>
<td>n-PBL + McF7</td>
<td>0.2 ± 0.01</td>
</tr>
<tr>
<td>n-PBL + Jurkat</td>
<td>0.6 ± 0.2</td>
</tr>
</tbody>
</table>

a Normal PBL were incubated in the presence of 1% (v/v) PHA and incorporation of [3H]thymidine was measured in 3 day microcultures containing 5 × 10^4 cells/well. PBL were tested before mixing with tumor cells. Immediately after mixing, cell suspensions were placed on SVG at 4°C and separated. Fractions containing lymphocytes were pooled, plated in microtiter wells and assayed for proliferative responses to PHA.
b n-PBL-T (normal peripheral blood T lymphocyte) fraction was obtained by the passage of mononuclear cells through a nylon wool column.
TABLE VI
RESPONSES OF TUMOR-INFILTRATING LYMPHOCYTES (TIL) TO PHYTOHEMAGGLUTININ (PHA) AFTER VELOCITY SEDIMENTATION AND DENSITY GRADIENTS

<table>
<thead>
<tr>
<th></th>
<th>[^H]thymidine incorporation cpm × 10^-3 ± SD</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Velocity sedimentation gradient</td>
<td>Density gradient</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>− PHA</td>
<td>+ PHA (SI)</td>
<td>− PHA</td>
<td>+ PHA (SI)</td>
</tr>
<tr>
<td>(1)</td>
<td>0.9 ± 0.2</td>
<td>1.9 ± 0.3 (2.1) ^a</td>
<td>4. 3.8 ± 0.3</td>
<td>2.7 ± 0.2 (1) ^b</td>
</tr>
<tr>
<td>(2)</td>
<td>1.6 ± 0.1</td>
<td>2.1 ± 0.1 (1.3)</td>
<td>5. 0.6 ± 0.1</td>
<td>0.9 ± 0.1 (1.5)</td>
</tr>
<tr>
<td>(3)</td>
<td>0.4 ± 0.05</td>
<td>0.6 ± 0.1 (1.5)</td>
<td>6. 0.6 ± 0.1</td>
<td>1.0 ± 0.01 (1.6)</td>
</tr>
<tr>
<td></td>
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<td></td>
<td>7. 0.2 ± 0.05</td>
<td>0.2 ± 0.04 (1)</td>
</tr>
</tbody>
</table>

Controls:
PBL + ENZ ^c 0.5 ± 0.1 26 ± 2 (52)
Tonsil + ENZ 0.3 ± 0.1 8.5 ± 0.7 (28)

^a The TIL-enriched fractions were obtained from three melanoma tumors. The TIL were plated at the concentration of 5 × 10⁵ cells/well and assayed for proliferative responses to PHA on day 3 of culture.
^b The TIL-enriched fractions (number 4–7) obtained from two melanomas, a colon carcinoma and an oesophageal carcinoma were studied.
^c As controls, normal PBL and tonsil lymphocytes were incubated with the same enzyme cocktail and under the same conditions used for digestion of tumors. The lymphocytes were placed on discontinuous density gradients, washed and plated as indicated above. Data represent mean cpm ± SD in triplicate cultures. Stimulation indices are given in parenthesis.

lymphocyte culture (SI = 8.8) were divided into two aliquots, one of which was layered on a velocity sedimentation, the other discontinuous density gradient. The MLC blasts, separated from small lymphocytes, were plated in the limiting dilution microcultures at 0.5, 2 and 5 cells/well in the presence of irradiated allogeneic spleen cells, 1% (v/v) PHA and IL-2-containing supernatants. Proliferating microcultures were scored on days 14–25 of culture and frequencies of proliferating cell precursors determined. Fig. 6 shows that the MLC blasts obtained by velocity sedimentation had a slightly better proliferating frequency (F = 0.36) than those obtained by density separation (F = 0.22). However, the difference was not significant (see 95% confidence limits in Fig. 6).

The same kind of analysis was applied to normal PBL that were mixed with Me8 melanoma cells and then separated from the tumor cells by velocity sedimentation. The frequency of proliferating cell precursors, which was 0.54 in PBL unexposed to tumor cells, decreased to 0.09 after sedimentation velocity in the presence of Me8 cells (Fig. 7). This reduction in cloning efficiency is significant (see confidence limits in Fig. 7) and indicates that inhibition of PHA-induced proliferation of lymphocytes exposed in vitro to tumor Fig. 6. Frequency of proliferating cells among MLC blasts separated on a discontinuous density gradient (A) and velocity sedimentation at unit gravity (B). The blasts were plated under limiting-dilution conditions at varying cell doses (0.5–5 cells/well) in the presence of irradiated allogeneic spleen cells, 1% PHA (v/v) and exogeneous source of IL-2. After 20 days of culture each microculture was scored microscopically for proliferation. Each point is based on a group of 42 microcultures. The regression lines were fitted to the data by the minimum X² method (see the materials and methods section). Frequencies of proliferating microcultures with 95% confidence limits are given at the bottom, left.
cells is demonstrable not only in populations but also at the level of single cells.

The TIL-enriched preparations obtained from human melanomas and separated on velocity sedimentation and/or density gradients were also studied by limiting-dilution analysis (Fig. 8). All three TIL fractions examined had low frequencies of proliferating cell precursors (0.009–0.01). Normal peripheral blood lymphocytes plated at the same dilutions in parallel cultures gave cloning frequencies ranging from 0.55 to 1.0 (data not shown) depending on the donor. In the case of the TIL 2 preparation (Fig. 8), cloning frequencies of the cells separated on the basis of their density or of their size were very similar (0.03 and 0.024, respectively). Therefore, we concluded that the inability of these cells to proliferate in response to PHA was not an artefact of separation methods. Rather, it was a characteristic exhibited by lymphocytes isolated from solid tumors as well as normal lymphocytes incubated in the presence of tumor cells.

Discussion

Separation of the TIL from human solid tumors presents a unique problem in that often only small biopsy specimens are available. For example, most surgical specimens of 40 human tumors we recently studied weighed < 3 g. The number of cells in disaggregated tumor tissue varied but was seldom larger than $10 \times 10^7$ and often much less. The other problem is that specimens arrive at any time and must be processed fresh and under sterile conditions. Therefore, a rapid, simple, and reproducible method that can be scaled down to accommodate small numbers of cells is needed. We chose to compare two such procedures, one based primarily on differences in size, the other in density, for their usefulness in obtaining human TIL.

Velocity sedimentation at unit gravity adapted to small gradient volumes and cell numbers performed well with artificially prepared mixtures of tumor cells and PBL. However, pathologic specimens, which are more heterogeneous, differed in
the recovery and purity of separated fractions (Table II). Basically, the purity of ‘large’ (tumor) cell fractions was excellent, while that of TIL was inferior. The recovery varied and in some cases approached 100%. The separation could be achieved in 3 h, but cell concentration and counts on individual fractions prolonged it to nearly 5 hours. The comparatively low purity of the TIL from melanomas could be related to the gradient capacity, cell streaming or cell aggregation (Shortman, 1984). Clearly, neither the gradient capacity nor the streaming limit, both of which are functions of the cell concentration, were exceeded (see Results), as defined in the preliminary mixing experiments we performed. The tumor-derived, enzyme-treated cell mixtures could be expected to aggregate, and even though they were filtered to reduce the frequency of clumps (MacDonald and Miller, 1970), the formation of cell aggregates during sedimentation cannot be excluded. Thus enzyme-treated tumor cells may tend to stick to the TIL and be retarded in their sedimentation. It seems likely that cell-to-cell interactions may explain the poor purity of our TIL fractions.

Discontinuous density gradients have been successfully used by Vose et al. (1977), Vose (1982) and Klein et al. (1980) for separations of infiltrating leukocytes and tumor cells (Yanagawa et al., 1985) from a variety of human tumors. Other investigators used one-step isopycnic density gradients (e.g., Stackpole et al., 1979) or discontinuous Percoll gradients (Kedar et al., 1982) for purification of mouse tumor cells. Even though Pretlow and Pretlow (1983) explicitly stated that density gradients are unsatisfactory for separations of cell mixtures in which densities of different cell types are likely to overlap, we resorted to these gradients as an easy, time-saving alternative to velocity sedimentation. Our comparisons of the two methods indicated that there was little difference in the cellular composition of TIL-enriched fractions recovered (Table III). The enrichment in TIL was also comparable and the viability of recovered TIL was always > 95%.

It has been suggested that centrifugation methods may lead to selective cell losses, particularly for the most rapidly sedimenting cells (Pretlow and Pretlow, 1983). A considerable loss in tumor cells occurred both on density and velocity sedimentation gradients. Since our central concern was with the TIL, the losses in purified tumor cells were acceptable provided the recovered cells remained viable and suitable for cryopreservation. For recovery of viable tumor cells, the discontinuous Percoll gradients used by Yanagawa and collaborators (1985) save time and thus may be more advantageous than velocity sedimentation at unit gravity. In terms of selective losses in the tumor-infiltrating mononuclear cells on density gradients, we compared cellular contents of the recovered TIL-enriched fractions to those determined in situ by immunohistologic techniques on sections of tumors and found them to be comparable (Whiteside et al., 1986). For example, both in situ and in recovered cell fractions, T11 + cells represented the major infiltrating elements, the proportions of T4 + and T8 + lymphocytes were similar, B cells and NK cells were rare, and the frequency of M1 + cells was comparably low (Whiteside et al., 1986). Thus, in our experience, the TIL-enriched fractions obtained by discontinuous density gradients accurately reflected the cellular composition of infiltrates in the stroma of human tumors studied.

Another major concern in cell separation methodology is the functional integrity of recovered cells. When normal PBL or MLC-generated blasts were passed through gradients, their functional responses were unimpaired as judged by responsiveness to mitogens (PHA, ConA) of cell populations and by frequency of proliferating T-cell precursors (PTL-P) determined in the limiting dilution assays (Table V, Fig. 6). However, a striking decrease of responses to mitogens in the normal PBL fractions separated from tumor cells following artificial premixing was observed at the population as well as the clonal levels (Table V, Fig. 7). This refractory behavior occurred irrespective of the type of gradient used for separation. Also, a similar lack of or decrease in responsiveness to lectins was observed when the TIL separated from many different tumors were tested (Miescher et al., 1986). Vose and Moore (1979) likewise observed decreased responsiveness to mitogens of the TIL populations. The reasons for it remain unclear, although it does not seem to be induced by the separation conditions, because normal PBL and MLC-induced blasts functioned
normally following enrichment on gradients. Nor was it induced by the putative removal of an accessory or 'helper' cell population, since it was not reversed by the addition of allogeneic spleen cells as feeders and IL-2 in cloning experiments. Rather, it appeared to be a characteristic feature of the TIL populations (Miescher et al., 1986) and, as indicated by the in vitro premixing experiments, could be related to effects exerted by tumor cells. Functional unresponsiveness of the TIL in cytotoxicity assays following their separation from human tumors has been reported by others (Vose et al., 1977; Totterman et al., 1980; Moy et al., 1985).

In conclusion, using either velocity sedimentation or discontinuous density gradients adapted to small volumes and relatively low numbers of cells, preparations enriched in TIL were obtained from human solid tumors. The two methods were comparable in terms of recovery, purity and cellular composition of the TIL fractions. Density gradients were found to be simpler, less time-consuming and more practical for routine preparations of TIL from clinical material than velocity sedimentation. Cells in the TIL-enriched fractions obtained by either of the two procedures had depressed responsiveness to mitogens, and this poor responsiveness appeared to be caused by the tumor milieu (Miescher et al., 1986) rather than separation procedures. These functional characteristics of the TIL were not related to tumor type, size or stage as determined by studies of enriched fractions obtained from many different tumors, both primary and metastatic.

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References