**ABSTRACT**

Successful therapy of tumors with lymphokine-activated killer (LAK) cells is presumably dependent on their cytolytic potential and their gaining access to the target cells through the microcirculation. The latter process involves dissemination through the microvessels, adhesion to the venular walls, and extravasation through them, all of which depend on the size and deformability of these effector cells. The aim of the present study was to measure the deformability of these cells quantitatively using a micropipet aspiration technique and to analyze the deformation data using a mathematical model which yielded parameters indicative of the rigidity of the cell membrane and the cytoplasm. Adherent rat LAK cells, consisting of a highly purified population of interleukin 2-activated large granular lymphocytes, with high cytotoxicity were obtained by a recently developed method. The deformability characteristics of fresh large granular lymphocytes (mean diameter, 7.2 μm), LAK cells (11.0 μm, fresh T-cells (6.6 μm), and concanavalin A-activated T-cells (9.7 μm) were compared. LAK cells were significantly less deformable than other cell types (about one-half at an aspiration pressure of ~25 mm of H2O (P < 0.001)). Cell deformability was independent of cell size and calcium content of the medium. Analysis of the data with the mathematical model suggested that both the cell membrane and the cytoplasmic factors contributed to the rigidity of LAK cells. This increased rigidity coupled with their large cell size may explain high retention of LAK cells in the lungs immediately after i.v. injection and a reduction in tumor targeting due to external radiation. Finally, these results suggest that LAK cell therapy might be enhanced by intraarterial injection into an organ infiltrated by tumor metastases.

**INTRODUCTION**

Adoptive immunotherapy with LAK cells offers a novel approach to the treatment of solid tumors (1-5). The efficiency of cancer therapy with LAK cells presumably depends on both cytolytic potential of these cells and their localization in tumor tissue. The latter parameter consists of several steps, including the distribution in the body, entrapment at the capillary entrance, adhesion to venular walls, and extravasation. Unfortunately, LAK cell therapy is currently limited by the toxic side effects on lung and other normal tissues.

Recent in vivo distribution studies have shown that 2 h after i.v. injection, a higher proportion of LAK cells are entrapped in the rat lungs than are LGL (47% versus 22%). Similarly, more AT cells are entrapped in the lungs than FT cells (40% versus 5%) (6-8). In addition to the presumed adhesion of these cells to the endothelial and/or cancer cells (9-13), it is presently unclear as to what mechanisms are responsible for this differential entrapment of activated cells. An understanding of the determinants of in vivo localization may suggest approaches for controlling or preventing normal tissue toxicity and, hence, may lead to improved strategies for adoptive immunotherapy of cancer.

Cell entrapment in vivo of various cell types is known to depend strongly on their size and deformability characteristics (14-17). Since LAK and AT cells have a relatively large diameter, and the former cells contain numerous cytoplasmic granules, these cells may have high resistance to flow. The in vivo distribution and in vitro morphological characteristics of these cells suggest the following hypothesis: LAK cells are more rigid than LGL; and AT cells are more rigid than FT cells.

To test this hypothesis, the deformability of these cells as well as of the WBC was quantified using a micropipet aspiration technique (14, 18). In this technique, negative pressure is suddenly applied to the cell membrane via a micropipet. The resulting deformation data are analyzed using an appropriate mathematical model to obtain viscoelastic coefficients for the membrane and the cytoplasm (19).

**MATERIALS AND METHODS**

**Cell Preparation.** LGL and FT cells were prepared from the spleens of male Fischer 344 rats (75 to 100 g; Taconic Farms, Germantown, NY). A single cell suspension in RPMI 1640 medium (Gibco) with 10% FCS was centrifuged on a Ficoll-Hypaque (Pharmacia Fine Chemicals, Uppsala, Sweden) density gradient to get mononuclear cells. Adherent monocytes, macrophages, and B-cells were depleted by passage through a nylon-wool column (Cellular Products, Buffalo, NY). LGL and FT cells were obtained as separate fractions by centrifugation on Percoll (Pharmacia) density gradients (20).

Purified LAK cells were prepared using a recently developed method (21). Briefly, LGL (2 x 10^6/ml) were cultured with human recombinant IL-2 (1000 units/ml; Cetus Co., Emeryville, CA) in 5% CO2/95% air at 37°C in a CM consisting of RPMI-1640, 10% FCS, 2 mM glutamine, 5 x 10^-4 M 2-mercaptoethanol, 1% minimal essential medium, nonessential amino acids, and antibiotics. After 2 days, 94 to 98% of IL-2-induced adherent cells had morphological and phenotypical characteristics of LGL and showed very high LAK activity in a standard 4-h 51Cr release assay. They were cultured for an additional 3 days with the filtered conditioned medium (0.2-μm Nalgene filter). Adherent LAK cells were removed from the culture flask surface in phosphate-buffered saline with 5 mM EDTA, washed with CM without IL-2, and stored up to 12 h at 4°C.

AT cells were obtained from FT cells (5 x 10^6/ml) by culturing with 5 μg/ml of Con A (Sigma) for 3 days (22). Contaminating LGL were removed by incubation with 40 μM l-leucine-methylster (Sigma) for 30 min at 37°C followed by Ficoll-Hypaque gradient centrifugation (8). WBC were obtained by centrifuging rabbit peripheral blood according to the procedure described in Ref. 19.

Experimental System. The experimental system is similar to the one previously used in our laboratory (18). Briefly, an inverted microscope (Diaphot; Nikon) was used for white light illumination, with heat-absorbing filters, a Turret condenser (numerical aperture, 0.52; working distance, 20.5 mm), a 100x oil immersion objective, and a 20x projection lens connected to a television camera (ITC-406; Ikegami). A video...
cassette recorder (IAG-6500; Panasonic) with a time code generator (WJ-810; Panasonic) and a television monitor (PM-205A; Ikegami) were used to record and display the images.

The pressure application system consisted of a damping chamber, a three-way manifold, two water reservoir bottles, and connecting tubes. The damping chamber partially filled with saline was adjusted to give a slight negative pressure (<0.5 mm H$_2$O) to the micropipet tip through a fine tube filled with saline. The step pressure was applied by rapidly opening the three-way manifold. The pressure was set by a manometer (precision, 10 μm; Daedel) and monitored by a transducer (precision, ±1%; Gould) connected to a chart recorder. The air/liquid volume ratios of both the damping chamber and two water reservoir bottles were adjusted to give a fast response (<3 ms at −25 mm of H$_2$O).

**Micropipet Aspiration.** The micropipet aspiration system consisted of a micropipet, micromanipulator (MO202; Narishige), and an observation chamber. Clean glass micropipets with 2- to 5-μm internal diameter (according to the cell size) were made using a micropipet puller (Model 720; Kopf) and filled with CM just before mounting on the micromanipulator. About 500 cells/mm$^2$ were suspended in the observation chamber. The chamber consisted of a plastic wall and glass coverslip bottom and was washed with saline and CM. A cell on the bottom of the chamber was attached to the micropipet tip by a slight negative pressure (0−) and picked up some distance. After each aspiration experiment, a slight positive pressure (+) was applied via three-way manifold to check and discard an adherent cell or cell fragments. The observation chamber was covered with a glass plate to reduce evaporation. The room temperature was 27 ± 2°C. The temperature increase in the chamber due to heating was less than 1°C/h. In case of poor contact or rotation of the cell (presumably due to the increased fluid volume in the micropipet by heating), the fluid was flushed out or a new micropipet was used.

The deformability of membrane and associated cytoplasm near to as well as remote from the nucleus was compared. Due to the small deformation of LAK cells in the physiological range of aspiration pressure (−5 to −25 mm of H$_2$O), −25 mm of H$_2$O were selected to quantify the deformation. The pressure application on each cell lasted less than 5 s and was not repeated more than 2 times to avoid cell activation or an irreversible change of cell structure under high aspiration pressure.

Due to the possible active changes in the cell shape and deformability in the Ca$^{2+}$-containing medium, the experiments were done with cells in both CM alone and CM supplemented with 0.15 g/100 ml of EDTA (CM + EDTA). The mean pH and osmolarity of both media were arranged in the physiological range (CM, pH 7.3 and 285 mOsmol/kg of H$_2$O; CM + EDTA, pH 7.43 and 291 mOsmol/kg of H$_2$O). Cells with abnormal cell morphology or depleted cytoplasmic granules were discarded. Each experiment was finished within 2 h, and the cell viability was checked using trypan blue stain.

**Data Analysis.** The cell deformation displayed on the television monitor (magnification, ×10,000 or ×20,000) was measured with a scale (precision, 1 mm). The outer edge of the dark interference band of the cell was used. The boundary which should be a smooth two-dimensional structure was discriminated by contrasting with optical flare. The cell deformation was defined as the difference between the length of dislocated cell portion, referred to as the tongue, and the spherical cell cap at $t = 0$ (Fig. 1). The initial television frame after application of a step pressure was found by an abrupt increase of $d(t)$. The, $d(t)$ was measured at 0.03-s intervals for 1.0 s. The error in measurement was less than 20% if the deformation was larger than 5 mm. Major source of error was the baseline shift, either due to the cell compression or due to the movement of the micropipet.

The standard solid viscoelastic model requires $d(t)$ to be less than 10% of the cell diameter (19). The deformation consists of an initial rapid, elastic response and a slow creep displacement, which is equivalent to a system made of two elastic elements, $k_1$ and $k_2$, and a viscous element, $\mu$ (19). These coefficients were obtained by fitting the model to data.

**RESULTS**

**Cell Morphology.** Morphology of each cell group was examined following cytospin deposition of cells and staining with Giemsa. LAK cells had larger cytoplasm than LGL and contained much more numerous cytoplasmic granules. AT cells were also larger than FT cells but had similar agranular cytoplasm. The diameter of each cell attached to the micropipet was measured before the aspiration experiment. In CM, the diameter (mean ± SEM) of LAK cells (11.0 ± 0.26 μm, $n = 41$) was significantly larger than that of LGL (7.2 ± 0.14 μm, $n = 58$). Also, the diameter of AT cells (9.7 ± 0.25 μm, $n = 45$) was larger than that of FT cells (6.6 ± 0.07 μm, $n = 49$). In CM + EDTA, similar differences were obtained between LAK (13.2 ± 0.19 μm, $n = 63$) and LGL (8.15 ± 0.23 μm; $n = 34$) and also between AT (11.0 ± 0.23 μm, $n = 31$) and FT (7.04 ± 0.11 μm, $n = 42$). These cells exhibited a significant increase in diameter (about 20%) compared to the cells suspended in CM alone. WBC had essentially the same diameter in CM (7.02 ± 0.19 μm, $n = 19$) and in CM + EDTA (7.01 ± 0.13 μm, $n = 27$).

**Cell Deformation.** $d(t)/D_p$ data of LAK, LGL, AT, FT, and WBC up to 1.0 s after −25 mm of H$_2$O step pressure are shown in Fig. 2. Deformability of the LAK cell was much smaller than that of a fresh LGL. Poking a LAK cell with the micropipet caused a small deformation which was reversible. LAK cells did not show increased adhesiveness to the micropipet in either CM or CM + EDTA. The location of the nucleus of LAK cells with respect to the micropipet did not affect their deformability. Analysis of the video tapes, however, suggests that while the location of the nucleus remained unchanged, the cytoplasm moved into the tongue during aspiration.

LGL, FT, and AT cells showed a slight ellipsoid change in cell shape within 1 to 2 s after the application of the step pressure, but LAK cells did not show a morphological change. Instead, they only showed an instantaneous shock-like vibration. These phenomena suggest a possible difference in the cytoskeletal structure between LAK cells and other cell groups.

LAK cells of all shapes (round, blunt, or shape with ruptured cell membrane and extrusion of the cytoplasm) showed only small deformations (<10% of the cell diameter) even several seconds after pressure application. However, occasionally (<10%) abnormal deformations with rapid, extensive protrusion and poor recovery after release of the pressure were observed. These cells exhibited morphological characteristics such as ballooning with few cytoplasmic granules or blurred cell membrane with large irregular granules, and sometimes showed the extrusion of cytoplasmic granules by pressure application. Similar abnormal morphologies were also observed in some AT cells, but not in LGL or FT cells. These cells were considered to be damaged and, hence, were discarded.
LAK CELL DEFORMABILITY

Fig. 2. Time course of normalized deformation, \( \frac{d(t)}{D_p} \), of LAK, LGL, AT, FT cells, and rabbit WBC in CM (A) and CM + EDTA (B). Points, mean; bars, SEM; numbers in parentheses, number of cells. Significant differences between cell types shown by specific marks are: *, LAK versus LGL, AT, FT, and WBC in CM (0.03 to 1.0 s, \( P < 0.001 \)); t, WBC versus LGL in both CM and CM + EDTA (0.1 and 0.3 s, \( P < 0.05 \)); t, FT versus LGL in both CM and CM + EDTA (0.03 to 1.0 s, \( P < 0.001 \)), and in CM + EDTA (0.1 to 1.0 s, \( P < 0.05 \)); †, FT and AT in both CM and CM + EDTA (0.3 to 1.0 s, \( P < 0.05 \)). No significant differences between media were observed except for WBC at 0.3 to 1.0 s (\( P < 0.05 \)).

Quantitation. LAK cells were about twice as rigid as LGL, AT cells, or FT cells (\( P < 0.001 \)). AT cells were also more rigid than FT cells (\( P < 0.05 \)). There was no significant difference between CM and CM + EDTA samples in either cell group. Correlation between either \( d(0.1)/D_p \) or \( d(1.0)/D_p \) and the cell diameter was not significant except for \( d(1.0)/D_p \) of LAK cells in CM (\( r = 0.31, P = 0.05 \)). Two-dimensional distribution of each cell group by cell diameter and \( d(1.0)/D_p \) showed rheological subpopulations of LAK and AT cells which were distinct from other subpopulations of LGL, FT cells, and WBC. Therefore, the product of \( D_c \) and \( [d(t)/D_p]^{-1} \), hereafter referred to as a resistance factor, was compared (Fig. 3). The value for LAK cells was 3-fold as large as for LGL and FT cells (\( P < 0.001 \)), and the value for AT cells was 1.5 times larger than that for FT cells (\( P < 0.01 \)). All these cells, in turn, were more rigid than WBC.

Viscoelastic coefficients gave results similar to \( d(t)/D_p \) (Fig. 4; Table 1). LAK cells have approximately twice as large \( k_1, k_2, \) and \( \mu \) as LGL, AT cells, or FT cells. AT cells had significantly larger \( k_1 \) and \( \mu \) than LGL or FT cells in CM. The difference between cells in CM and CM + EDTA was only significant in \( k_1 \) of FT cells. The ratio of viscoelastic coefficients between fresh and activated cells (LAK/LGL and AT/FT) showed a different effect of activation on the cell structure. LAK/LGL had a ratio of about 2 in all viscoelastic coefficients, but AT/FT had the ratio of less than 1.5 (Table 1). These results demonstrate that LAK cells have increased values of \( k_1, k_2, \) and \( \mu \), but AT cells have increased values of only \( k_1 \) and \( \mu \).

DISCUSSION

The objective of the present study was to measure the deformability of LAK cells in vitro and to evaluate its implications for their distribution in vivo. Deformability is dependent on multiple parameters, including cellular morphology, cytoskeleton structure, IL-2 concentration, discharge of cytoplasmic granules, and Ca\(^{2+}\) content. LAK cells were found to be about half as deformable as LGL and FT cells. AT cells were less deformable than FT cells, but were not as rigid as LAK cells (Fig. 2). The viscoelastic coefficients of LAK cells showed that both the cell membrane and the cytoplasm contributed to the decreased deformability (Table 1). These results on the increased rigidity of LAK and AT cells are in agreement with the hypothesis that the initial cell entrapment in the lung after i.v. injection is determined by cell rigidity and diameter (15, 16).

WBC from rabbit peripheral blood were more deformable than any other cell types except for FT cells. The viscoelastic coefficients of WBC at ~5 mm of H\(_2\)O aspiration pressure had values similar to those of human peripheral blood measured at a lower pressure range (~2.8 to 9.0 mm of H\(_2\)O) (14). However, when measured at ~25 mm of H\(_2\)O aspiration pressure, the value of viscoelastic coefficients of rabbit WBC was higher than that measured at ~5 mm of H\(_2\)O.

In contrast to the homogeneous appearance of LGL, LAK cells were heterogeneous in cell size and the content of cytoplasmic granules, presumably due to difference in cell maturation and stages in the cell cycle. Two morphological types of human LAK cells have been described, which vary in the morphology of their cytoplasmic granules (23, 24). In the present study with rat adherent LAK cells, we occasionally (<10%) observed unusually well-deformable LAK cells with few
cytoplasmic granules. In separate experiments, preparations of cells were depleted of LGL by L-leucine methylester resulting in a pure population of cells that are precursors to natural killer cells (LAL) (25). LAL were compared with normal LGL, but no significant difference in the deformability was observed between them. We did not observe any changes in cellular integrity of morphologically normal cells due to aspiration at -25 mm of H2O except for the occasional small tear of the cell membrane at the deformed cap.

The diameter and deformability of LAK cells were poorly correlated in spite of diverse distribution of the cell diameter due to growth and differentiation. This observation suggests that it may not be possible to select LAK cells a priori with specific deformability on the basis of cell size. In addition, a correlation was not observed between viscoelastic coefficients and cell diameter in either cell group suspended in CM or CM + EDTA. It seems likely that the in vivo ability of LAK cells to pass through capillaries depends on their deformability as well as their diameter. Therefore, the product of Dc and [d(t)/Dp] -1, referred to as a resistance factor, was compared (Fig. 3). The value for LAK cells was 3-fold larger than that for LGL and FT (P < 0.001), and the value for AT cells was 1.5 times larger than that for FT cells (P < 0.01).

Leukocytes (same as the WBC in the present study) in Ca2+-containing medium are known to develop pseudopods which have a decreased deformability compared with that of passive cells in Ca2+-depleted medium (26). We also found a significant difference in deformability of WBC suspended in CM and in CM + EDTA at 0.3 to 1.0 s (P < 0.05; Fig. 2) (measurements were made only on the nonpseudopod area of the cell surface). In contrast to WBC, we could not find any difference in deformability of other cell types (LAK, LGL, AT, and FT cells) in different suspending media. Therefore, the short-term deformation (<5 s) of the latter cells appears to be a passive, mechanical response, not a Ca2+-dependent biological process.

The deformability of T-cells was reported to be less than that of neutrophils, presumably due to the membrane villi and large nucleus in the former cells (14). In contrast, a difference in deformability was seen between T- and B-cells in spite of similar cell size and surface characteristics (27). Based on the viscoelastic coefficients, we propose that the difference in deformability between AT cells and FT cells (AT/FT) and between LAK cells and LGL (LAK/LGL) in the present study is due to morphological and functional changes in both the cell membrane and the cytoplasm induced by Con A and IL-2, respectively.

Poorly deformable myeloblasts of acute myelocytic leukemia with a large spheroidal nucleus are known to cause symptoms in human patients related to the microvascular damage due to decreased blood flow and poor tissue oxygenation (28, 29). Rabbit solid tumor cells with decreased deformability are also known to stick for a long time in the rabbit capillaries (30, 31). LAK cells are not as numerous as leukemic cells and, therefore, may not directly cause microcirculatory ischemia by entrapment, but upon adhesion they may produce damage to endothelial cells with a consequent increase in microvascular permeability (13). When LAK cells are maintained in culture with IL-2, they fluctuate in their adhesiveness as well as their shape (21, 32; Footnote 6). Analogous changes in adhesiveness and deformability may occur in LAK cells in vivo to permit escape through capillaries.

### Table 1: Viscoelastic coefficients

<table>
<thead>
<tr>
<th>Cell types</th>
<th>k1 (dyn/cm²)</th>
<th>k2 (dyn/cm²)</th>
<th>μ (dyn/s/cm²)</th>
<th>Time constant (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CM LAK (41)</td>
<td>6713 ± 433³</td>
<td>7938 ± 654³</td>
<td>2733 ± 204³</td>
<td>0.764 ± 0.017³⁴</td>
</tr>
<tr>
<td>CM LGL (50)</td>
<td>2637 ± 145⁴</td>
<td>3888 ± 190⁴</td>
<td>1353 ± 71.1⁴</td>
<td>0.892 ± 0.020³⁴</td>
</tr>
<tr>
<td>CM + EDTA AT (45)</td>
<td>3878 ± 238⁴</td>
<td>4346 ± 244⁴</td>
<td>1663 ± 95.3⁴</td>
<td>0.852 ± 0.023⁴</td>
</tr>
<tr>
<td>CM + EDTA FT (49)</td>
<td>2520 ± 138⁴</td>
<td>3829 ± 175⁴</td>
<td>1215 ± 57.6⁴</td>
<td>0.839 ± 0.019⁴</td>
</tr>
<tr>
<td>WBC (19)</td>
<td>2004 ± 159⁴</td>
<td>2793 ± 336⁴</td>
<td>952 ± 93.0⁴</td>
<td>0.847 ± 0.042⁣</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, number of cases.
* LAK versus LGL, AT, FT, and WBC (P < 0.001).
* Mean ± SE.
* P < 0.01.
* P < 0.05.
* P < 0.001.
* k₃ of CM and CM + EDTA is significantly different (P < 0.001).

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Fig. 4. Simulation of the cell deformability by standard viscoelastic model and the viscoelastic coefficients (k₁, k₂, and μ) of LAK and LGL (A) and of AT and FT cells (B). For details of the model, see the text.
from capillaries in a manner similar to neutrophils' (33).

The tumor microvessels have sluggish blood flow; dilated, saccular, and partially collapsed vessels; and poorly developed basement membrane (17). Therefore, after the LAK cells enter the tumor microcirculation, their adhesion and extravasation may be much easier in tumors compared to several normal tissues. Unfortunately, following the conventional systemic injection, very few LAK cells arrive at the sites of tumor growth and, hence, a relatively small fraction of total cells injected localizes in tumors (2). A decrease in tumor interstitial pressure caused by radiation may open up partially collapsed tumor vessels (34) and may further reduce entrapment of LAK cells and/or facilitate escape of entrapped LAK cells. Therefore, external beam radiation may lead to a reduction in uptake of LAK cells by tumors (35). One possible method of increasing the uptake of LAK cells by a tumor would be the local arterial injection of LAK cells in the organ infiltrated by tumor metastases. This approach would bypass the initial entrapment of LAK cells in other normal organs (e.g., lungs, liver, and spleen) and might take advantage of the peculiar nature of the tumor microcirculation (17). This hypothesis remains to be tested by intraarterial injection of LAK cells and by direct observation of their behavior in the normal and tumor microcirculation (16).

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