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Cell Behavior Analysis to Evaluate Proliferative Potentials of Human Lymphocytes Expanded and Activated for Therapeutic Use

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The cluster designation 3-lymphokine activated killer (CD3-LAK) culture was conducted using human lymphocytes obtained from different donors. It was found that donor-dependent variances existed in terms of lag time and minimum doubling time, which were process parameters for comprehending the proliferative potentials of cells in an early phase with weak growth and in a subsequent phase with active growth, respectively. To correlate these variances with culture performances, cellular behaviors were estimated by constructing a custom-made observation tool that can capture and process images of culture surfaces. The tool enabled us to determine time-lapse changes in an average projected cell area and the frequency of cell aggregates during the culture in a noninvasive manner. It was found that linear relationships were obtained both between lag time and average projected cell area, and between minimum doubling time and formation rate of cell aggregates, irrespective of culture performance fluctuation depending on each donor state. It is concluded that the developed tool is helpful for operating CD3-LAK culture while monitoring the state of human lymphocytes.

[**Key words:** cell therapy, human lymphocytes, image analysis, cellular behaviors, cell aggregates]

Immuno-cell therapy has become of interest as clinical treatment for cancer patients by the administration of lymphokine-activated killer (LAK) cells (1–4). In cell processing, a marginal amount of autologous blood is collected from a patient to obtain starter cells, and starter cells are activated by anti-CD3 antibody stimulation to initiate cell division. Then, the expansion of LAK cells is conducted with interleukin-2 doping for several weeks, and harvested LAK cells are ultimately administered into the patient. In practical processing, starter cells possess population heterogeneity in proliferative potentials depending on each patient state such as physical condition, age and gender. This population heterogeneity causes variances in cellular behaviors such as activation, cell division and aggregation during culture, frequently leading to process instability, that is, variation in the final number of cells and vitality. Mason *et al.* (5) pointed out that the monitoring and control of cultures for autologous cells are labor- and cost-intensive, and still it is in practice required to understand the properties of respective cultures in a custom-made manner. Microscopic observation can be a useful tool for assisting operators who conduct monitoring and prehension of cellular states during cultures. In conventional cell processing, however, the understanding of cellular states largely relies on the know-how of experienced operators. In addition, a fluctuation in culture conditions like temperature and oxygen tension affects cell proliferation (6, 7), and therefore an *in situ* and noninvasive observation system is desired.

In our previous study (8–10), an observation tool was developed to perform time-lapse tracing of individual cells. The machinery, in which image capturing positions can be changed by electrical stages in an arbitrary manner, enables static culture without moving a vessel, and therefore enables the observation of the dynamic behavior of anchorage-independent cells (11). In the present study, dynamic cellular events in a population of immunocytes were examined using a custom-made observation tool with some modifications. In addition, cellular swelling and cell aggregate formation were correlated with lag time in the lag phase and with minimum doubling time in the early growth phase, respectively, to evaluate growth potential in a whole population of cells during cluster designation 3-lymphokine activated killer (CD3-LAK) culture.

This research was approved by the ethical committees of both Medinet Co. and Seta Clinic, Japan. Healthy adult volunteers donated human lymphocyte cells with informed consent. PBMCs (peripheral blood mononuclear cells) and blood plasma were harvested using a blood collecting tube (BD Vactainer CPT; Becton Dickinson, Franklin Lakes, NJ, USA), followed by centrifugation (1710×*g*, 25°C). The PBMCs were washed twice with physiological saline, and then subjected

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to CD3-LAK culture at 37°C in a 5% CO_2 atmosphere in a 225-cm^2 T-flask (Suspension Culture Flask 800; Sumitomo Bakelite, Tokyo). At the bottom surface of the flask, an anti-CD3 antibody was displayed. Due to the limited volume of blood harvest from each donor, the culture was started using a single flask. The initial concentration of lymphocytes, determined by cell counting using a hemocytometer, was fixed at $X_0 = 5.0 \times 10^6$ cells per a flask. The incubation of cells was initiated in 50 ml of serum-free medium containing 175 IU/cm^3 interleukin-2 (KBM400; Kohjin Bio, Sakado) and 8% autologous blood plasma, and 150 ml of the fresh medium was added to the flask at $t = 72$ h of culture time. At $t = 120$ h, 200 ml of the fresh medium was further fed to the flask, and half of totally 400 ml culture was taken for introduction into new antibody-free flask.

The cell suspension in the flasks was sampled to determine the number of lymphocytes at a given culture time, by direct cell counting on a hemocytometer. The number of cells was monitored to estimate lag time and doubling time in the course of culture. For the time-lapse observation in the lag phase and early growth phase using the observation tool, culture with 12-well plates (Suspension multiwell plate; Sumitomo Bakelite, Tokyo) was conducted in a chamber kept under the same culture conditions as mentioned above. The images in each culture well were captured at four positions at the bottom surface every 12 h using a high-resolution camera (CSB4000CL; Toshiba Teli Co., Tokyo) with an objective lens. Here, objective lenses of 40 and 20 magnifications (NPLM40XOB; Union Optical, Tokyo; and LCPLANFL20-XPH; Olympus, Tokyo, respectively) were employed for observing the cell area in the lag phase (captured area, 0.12 mm^2) and cell aggregation in early growth phase (captured area, 0.38 mm^2), respectively. The camera was mounted on a stage that can be electrically driven in the X-, Y- and Z-directions to take arbitrary positions at the bottom surface of the well and to adjust each focus on target cells at each position.

The projected areas of an individual cell and aggregate in the cultures were determined by extracting cell and aggregate edges using a line-drawing tool of software (IMAQ Vision Builder; National Instruments, Austin, TX, USA). Average projected cell area was calculated using single cellular objects on four images. The aggregate was defined as a cellular object with a projected area of more than 100 μm^2, and the frequency of cell aggregates was obtained as {100 × (counts of aggregates)/(counts of single cells and aggregates)}. The formation rate of aggregates was estimated by averaging the changes in the frequency for $t = 24$–72 h, which means the variation in cell aggregate frequency per hour.

CD3-LAK cultures were conducted to determine the growth profile and behavior of lymphocytes obtained from dozen donors, nominally designated a to l. Figure 1 shows the representative change in the total number of cells with culture time together with selected snapshots of cell behaviors. The profile typically exhibited the two phases accompanied with relatively weak and active cell proliferations. Hence, apparent doubling time, t_d, at a given culture time was estimated by the following equation.

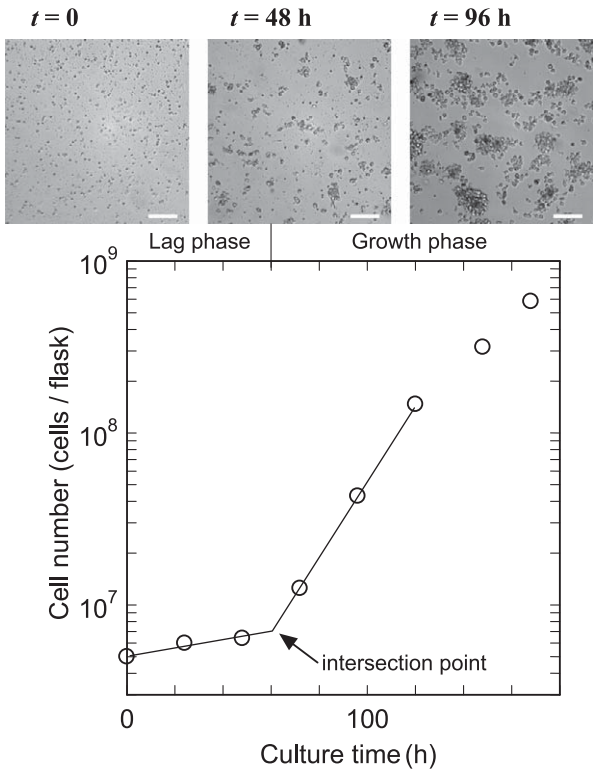

FIG. 1. Typical time profile of cell growth and snapshot images showing cell behaviors in CD3-LAK culture of lymphocytes (from donor a). The white bars on the images show 100 μm.

$$t_d = \frac{\ln 2 \cdot (t_2 - t_1)}{\ln X_2 / X_1} \quad (1)$$

where X_1 and X_2 are the number of cells at t_1 and t_2, which correspond to the times of current and foregoing measurements during the culture, respectively. The boundary between these phases was determined as an intersection obtained by extrapolating linear lines in the respective phases, and lag time was estimated to be the culture time until the intersection point. In the culture shown in Fig. 1, the lag time and minimum doubling time were evaluated to be 60.2 h and 10.6 h, respectively. In the lag phase, the cells were observed to swell, and some of the swelling cells initiated division and migration in the late lag phase, during which an increase in the number of cells was negligibly small. In the growth phase, active migration was observed to cause the coalescence between cells. Simultaneously, the cell division promoted clustering, leading to the development of cell aggregates. Table 1 summarizes the lag times and minimum doubling times in the CD3-LAK cultures of lymphocytes from donors a to l. The culture performances presented a significant variance in both lag time and minimum doubling time. The lag time and minimum doubling time were in the ranges of 53.6 to 144.6 h (S.D.±29.45 h) and 7.8 to 21.6 h (S.D.±4.75 h), respectively, indicating that these parameters are largely affected by the type of starter cell source.

To analyze the relationship between the morphological change and growth potential, the projected area of each cell was determined during the lag phase in each culture. Figure 2a shows the time profiles of average projected cell area in the