Lymphokine-activated Killer Cells: Culture Conditions for the Generation of Maximal in Vitro Cytotoxicity in Cells from Normal Donors

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ABSTRACT

The current method for generating lymphokine-activated killer (LAK) cells for use in human clinical trials is labor intensive and expensive. Therefore, we altered culture conditions to determine whether LAK cells with enhanced lytic activity could be generated. Culture of normal human peripheral blood leukocytes for 7 days generated LAK cells with 4-fold more lytic activity than culture for 3 days. Although cell viability over this 7-day period dropped from 94% on Day 3 to 73% by Day 7, the recovery of cells from culture increased from 61 to 106%. If cells were exposed to CO₂, lytic activity was further enhanced by up to 30-fold. Culture at a density of 1 or 2.5 x 10⁶ cells/ml caused no difference in cell viability, recovery, or LAK activity when cells were cultured for up to 4 days; however, when cells were cultured for longer times, an initial density of 1 x 10⁶ cells/ml yielded maximal LAK activity. Several commercially available serum-free defined media as well as human serum albumin supported LAK cell activation comparable to serum-containing media over a 4-day culture period. One defined medium, AIM V, supported LAK cell activation over a 7-day period even when cells were cultured at a density twice as high (2 x 10⁶ cells/ml) as cells cultured in serum-containing medium. The results demonstrate that simple manipulation of human LAK cell culture conditions generates cells with greatly enhanced lytic activity and that serum-containing medium may not be necessary for generating LAK cells under the current clinical protocols.

INTRODUCTION

The precise mechanisms that mediate the antitumor effects of LAK cell therapy are not known; however, LAK plus IL-2 therapy is clearly more efficacious than therapy with IL-2 alone (1). The adoptive transfer of such cells plus IL-2 has been shown to mediate the regression of pulmonary and hepatic metastases from a variety of established murine tumors, including the B16 and M3 melanomas; the MCA-101, 105, and 106 syngeneic sarcomas; the MC-38 murine colon adenocarcinoma; and the 1660 murine bladder carcinoma (2-5). More recently, combined therapy with LAK cells and IL-2 in humans was demonstrated to cause marked tumor regression in 11 of 25 patients with advanced metastatic cancer (6).

The results demonstrate that simple manipulation of human LAK cell culture conditions generates cells with greatly enhanced lytic activity and that serum-containing medium may not be necessary for the generation of LAK cells under the current clinical protocols.

A major limitation to large scale application of LAK cell therapy is the expense and limited availability of human AB serum. Given the problems associated with the use of serum, such as lot variability, expense, and possible disease transmission, the use of a totally defined, serum-free medium for the generation of LAK cells is desirable. We therefore examined LAK cell generation under a variety of culture conditions to maximize lytic activity and tested the suitability of serum-free defined media for culture of LAK cells.

The results demonstrate that (a) LAK cell cytotoxicity is much higher after longer times in culture (up to 8 days), when there is a slight decrease of cell viability but an increase in cell recovery; (b) the activity of LAK cells generated in the presence of CO₂ is much greater than that of cells grown in its absence, even at suboptimal times in culture; (c) LAK cells can be cultured at a density of either 1 or 2 x 10⁶ cells/ml for 4 days but only at the lower density for 7 days; and (d) commercially available defined serum-free medium can support the growth and differentiation of LAK cells to a degree comparable to that of serum-containing media.

MATERIALS AND METHODS

Interleukin 2. Human recombinant IL-2 was generously supplied by the Cetus Corporation, Emeryville, CA. This IL-2 had a specific activity of 3 to 4 x 10⁸ units/mg protein. The IL-2 was diluted so that a unit corresponds to a Biological Response Modifiers Program unit measured as described (8). Maximum LAK activity was observed with 1000 units/ml.

Generation of LAK Cells. Normal healthy volunteers were leukapheresed using a 2-needle leukapheresis procedure on a Celltrifuge II leukapheresis apparatus (Fenwal Laboratories, Deerfield, IL), as previously described (9). The final volume of the leukapheresis pack was 200 to 300 ml. An equal volume of HBSS without calcium and magnesium was added to the bag, and 35 ml were layered over 15 ml of lymphocyte separation medium (Litton Bionetics, Kensington, MD); dilution and gradient preparation took less than 10 min. Gradients were centrifuged at 900 × g for 15 min at room temperature, and the separated mononuclear cells were removed, pooled, and washed twice with HBSS. Cells were resuspended by gentle pipetting with a 10-ml pipet. During the processing of the cells, care was taken to promptly continue the next step of processing, so that at no time did the cells sit in contact with lymphocyte separation medium or as a dry pellet for more than a minimal period of time. Cells were incubated at the indicated density in RPMI 1640 containing 2% heat-inactivated human AB serum, 50 units/ml penicillin, 50 μg/ml streptomycin sulfate, 50 μg/ml gentamicin sulfate, and 2 mM glutamine with 1000 units/ml IL-
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2 (Cetus). The human AB sera were screened for hepatitis, HTLV III, bacteria and Mycoplasma by the vendors and further selected on the basis of their ability to generate LAK cell activity and reproducible cell yields from culture. During our screening we found no significant difference between human AB, A, and plasma-derived AB serum (10).

Complete medium (without IL-2) was obtained from either M. A. Bioproducts (Walkersville, MD) or GIBCO (Grand Island, NY). There was no significant difference in the ability of either product to support the growth and differentiation of LAK cells. Cells were grown in either roller bottles or flasks as described in the figure legends and harvested at various times for the measurement of LAK activity.

The HB series of defined media were obtained from DuPont (NEN Research Products, Boston, MA); AIM V medium was a preproduction sample provided for experimental testing by GIBCO/BRL (Grand Island, NY). All were reconstituted as recommended by the supplier. Human serum albumin (25%) was from the Red Cross.

Cell Counts. Nonadherent cells were harvested and resuspended in HBSS containing trypan blue. Cells were counted with a hemocytometer; counts excluded red cells and cell debris. The concentration of effector cells in cytotoxicity measurements (see below) thus reflects only viable nonmononuclear cells.

Measurement of Cytotoxicity. LAK cells generated as described were cytolytic against a large spectrum of human tumor cell lines and freshly isolated human tumors (data not shown) as described by others (11, 12). Due to the variable and often low viability of frozen tumor targets, the relatively high spontaneous release of radioactivity, and the variability among freshly isolated tumor cell suspensions, the Daudi cell line was routinely used to compare LAK cell activity generated under various culture conditions. Daudi is a B-cell line derived from a patient with Burkitt's lymphoma (13) that is resistant to lysis by natural killer cells. We have found no circumstances in which LAK cell activity measured against the Daudi cell line was falsely positive when compared to lysis of fresh tumor.

LAK activity was measured in a 4-h 51Cr release assay. The target cells were used while in the logarithmic growth phase and labeled for 1 h at 37°C as described (14). Assays were performed in round-bottomed 96-well plates in 200-μl volumes containing 5 × 10^6 target cells. Effector:target ratios of 16:1, 8:1, 4:1, and 2:1 were routinely used. Percentage of lysis ranged from 10 to 80% at these ratios. Spontaneous release of 51Cr from the labeled target cells was always <20% of total counts, and usually <10%. The cells were incubated for 4 h at 37°C in an atmosphere of 5% CO₂ in air (14).

At the end of the 4-h incubation period, the supernatant containing the released 51Cr was harvested with a Titertek harvesting system.

\[ \text{% of release} = \frac{\text{cpm released by cells during incubation}}{\text{total cpm incorporated to cells}} \times 100 \]

The percentage of specific cytotoxicity was calculated as the percentage of 51Cr released in the experimental group minus the percentage of release in the medium control.

LAK activity is expressed in LUs which are defined as the number of cells which cause 20% lysis of 5 × 10^6 cells over the range of effector:target ratios used and calculated according to the method of Pross et al. (15).

RESULTS

During all of these experiments, care was taken to process the cell samples gently and promptly. Cells were always seeded in prewarmed culture media and placed in the incubator within 1.5 h of the arrival of the sample to the laboratory. When cells were cultured in roller bottles, optimal pH (pH 7.4) was easily maintained. However, when cells were cultured in volumes of less than 1 liter, in either roller bottles or flasks, there was a rapid loss of CO₂ (alkalinization) from the media. To control these pH changes, flasks were preequilibrated overnight at 37°C, 5% CO₂, and media were preequilibrated in the flasks for at least 2 h prior to seeding the cells to prevent alkalinization of the media. Such precautions were particularly important when using defined media which lack serum-buffering capacity.

Despite all of these precautions, the activity of LAK cells generated from normal donors differed significantly from one donor to another. Fig. 1 shows the lytic activity of LAK cells generated as described (7) from 15 different normal donors. Cytotoxicity was measured after various days in culture as indicated. Although there was great variability among donors, the average lytic activity of cells cultured for 5, 6, or 7 days (131, 170, and 132 LU/10^6 cells, respectively) was significantly higher (3-fold) than the activity of cells cultured for 3 and 4 days (45 LU/10^6 cells). Even after 10 days in culture, the average lytic activity (110 LU/10^6 cells) was still significantly greater than that after 4 days of culture.

During culture for more than 4 days, significant differences were observed in both cell viability and cell density (Fig. 2). For the first 4 days of culture, cell viability remained high (94 ± 2.6%). However, by Day 5 of culture, viability decreased to 83 ± 5.5% and was 73 ± 4.4% by Day 10 of culture. Cell density, on the other hand, increased significantly during this time. Cells were initially seeded at a density of 1 × 10^6 cells/ml. During the first 24 h of culture, many cells adhered to the culture vessel so that by Day 1 of culture, cell density averaged 0.61 ± 0.09 × 10^6 viable cells/ml. There was no significant increase in nonadherent cell density until Day 7 when cell density increased somewhat, and by Day 10, density averaged 1.06 ± 0.12 × 10^6 cells/ml. Therefore, viable cell number/ml remained constant for up to 7 days and gradually increased to almost double initial values by Day 10. Cells could be further expanded by the addition of fresh medium after 10 days of culture (data not shown). However, the experiment was terminated at Day 7 to allow direct comparison of lytic activity without complications arising from nutrient deficiency and differences in cell density.

A number of different culture conditions were tested to maximize LAK cell activation (Fig. 3), including culture for longer times, stationary versus rotating roller bottles, and the presence and absence of CO₂. To normalize for changes in cell viability and number during culture, data are expressed as lytic units per ml. Under the current clinical protocol (7), cells are activated for 3 to 4 days in rotating roller bottles in the absence of CO₂.
of CO₂. As seen in Fig. 3, culture for more than 3 days under these conditions significantly enhanced lytic activity from 10 to 20 LU/ml. Although the lytic activity of cells cultured in stationary and rotating roller bottles for 3 to 4 days was not significantly different, the lytic activity of cells cultured in stationary roller bottles for 7 days was significantly greater than that of cells cultured in rotating roller bottles.

However, the most dramatic increases in LAK activity were observed, under all conditions, when cells were grown in the presence of CO₂. When cells were cultured for longer times, up to 7 days, the increase in LAK activity in the presence of CO₂ was even greater. The lytic activity of cells cultured in T150 flasks was much greater than that of cells cultured in roller bottles; lytic activity after 7 days culture in T150 flasks was 1.5 times that of cells cultured in roller bottles in the presence of CO₂ and 30 times greater than that of cells cultured in the absence of CO₂. The increased total lytic activity of cells cultured in the presence of CO₂ reflects an increase in specific lytic activity as well as an enhanced recovery of cells from culture. None of these culture conditions significantly affected cell viability.

The effect of cell density on LAK cell activation was also tested. Cells were seeded at 1, 2.5, or 5 × 10⁶ cells/ml, and LAK activity was tested after 3, 5, or 7 days of culture (Table 1). Clearly, 5 × 10⁶ cells/ml are too high a density, as LAK activity was much reduced compared to that of cells grown at lower densities at all times tested. The effect is likely due to cell crowding and nutritional deficiency, since cells had a tendency to clump and cell viability was reduced from 80 to 65%. Total cell recovery was also reduced from 60 to 23% by Day 5 and from 86 to 29% by Day 7, when cells were cultured at 5 × 10⁶/ml. In contrast, the specific lytic activity and recovery of cells seeded at 2.5 × 10⁶ cells/ml were not significantly different from those of cells seeded at 1 × 10⁶ after 3 days of culture. However, after 5 and 7 days of culture, the activity and recovery of cells grown at the higher density were much less. Therefore, for 3- or 4-day LAK cell activation, culturing at 2.5 × 10⁶ cells/ml is equivalent to the lower density, but if cells are cultured for longer times, lower initial density results in better lytic activity. The data from Table 1 again demonstrate that culturing cells beyond the standard 3 days results in increased lytic activity.

The ability of human serum albumin, serum-containing media from 2 companies, and several commercially available serum-free defined media to generate LAK cells was examined. Cell recovery from culture and lytic activity are expressed as fold parallel control values (cells cultured in RPMI with 2% human AB serum) to allow direct comparison of several experiments. As seen in Table 2, there was no difference in the ability of 2% human AB serum-containing media from either GIBCO or M. A. Bioproducts to support LAK cell activation or recovery from culture. All of the defined media supported the activation of LAK cells to an extent comparable to serum-containing media when cells were cultured for 4 days. However, with the exception of AIM V, the other defined media did not support increased lytic activity over a 7-day activation period, as did serum-containing media. Interestingly, 2% human serum albumin was not significantly different from the defined media under these conditions. After 7 days of culture, the total lytic activity (percentage of recovery × LU/10⁶ cells) of cells cultured in the HB series and human serum albumin was reduced to 17 to 45% of that of cells cultured in serum-containing media, while that of cells cultured in AIM V medium was increased to 208% control values. The recovery of cells cultured in defined media was slightly reduced compared to that of cells cultured in serum-containing media, but there was no significant difference in cell viability.
high cell viability (greater than 90%) and cell yields from culture as well as consistent LAK activity in vitro.

In addition to careful quality control in handling the cells, simple alteration of culture conditions can result in cells with 30-fold more lytic activity in vitro (Fig. 3) than cells grown under conditions presently used for clinical trials (7). The most important factors seem to be time of culture and exposure to CO₂, since cell yields (Fig. 2) and specific LAK activity (Fig. 1) greatly increased with time in culture especially in the presence of CO₂. Although cell viability decreased somewhat, 94% of 0.61 × 10⁶ cells/ml (Day 3) is less than 73% of 1.06 × 10⁶ cells/ml (Day 7), so this longer culture time actually yields more viable cells with increased specific cytolytic activity. The positive effect of CO₂ is also greater over longer periods of culture (Fig. 3). Although LAK activity generated in flasks was greater than that of cells grown in roller bottles (in the presence of CO₂), the volumes of cells generated with the current clinical protocol make the routine use of flasks impractical, unless larger culture vessels are found to be suitable. However, if it is assumed that enhanced lytic activity in vitro correlates with enhanced tumor regression in vivo, it may not be necessary to culture such large numbers of cells since cells cultured in flasks are 30 times more lytic than cells cultured in capped roller bottles. Additionally, since there is no significant difference in LAK cell recovery or activity if cells are cultured at 1.0 or 2.5 × 10⁶ cells/ml for 3 or 4 days, it is feasible to culture patients’ cells at this higher density.

Comparison of lytic activity (Fig. 1), cell viability, and cell recovery (Fig. 2) of LAK cells generated from normal volunteers over time in culture suggests that an event occurs after 4 days of culture in IL-2 which affects all of these parameters. The average lytic activity (LU/10⁶ cells) increases from 40 on Day 3 to 49 on Day 4 to 131 on Day 5. Also at Day 5, cell viability begins to decrease, averaging 85% as opposed to 95% on Days 3 and 4. After 6 days in culture, nonadherent cell number begins to significantly increase and continues to increase for up to 10 days in culture. Due to the mixed nature of the culture, it is difficult to determine which subpopulations of cells are expanding or dying out. It is possible that an initial differentiation event occurs within the first 72 h of culture followed by an expansion of the LAK cell subpopulation. This speculation is supported by the observation that specific LAK activity significantly increases after 4 days in culture. Studies are in progress to examine these questions.

The use of defined media for LAK cell activation would provide both safer and less expensive conditions for large-scale cultivation of LAK cells. As noted above, careful control of pH is even more important when growing cells in defined media; not only is alkaline pH toxic to human mononuclear cells, but some of the defined media components are sensitive to pH changes. Several defined media have been described which support the proliferation and function of human lymphoid cells (16, 17). More recently, a defined medium which supports the induction of LAK cells has been described (18). However, the proliferation and lytic activity of cells cultured in this defined medium were much less than those of cells cultured in medium containing human sera. In contrast, it has been reported that LAK activity could not be detected in cells grown in defined Medium HB104 (7), while other data (19) suggest that HB104 is actually better than human AB serum in maintaining long-term LAK activity. However, cell viability, recovery data, and assessment of media pH were not presented in these studies. Our own studies demonstrate that several commercially defined media including HB104, or human serum albumin used as a
serum supplement in RPMI 1640, can support LAK cell viability and lytic activity comparable to serum-containing medium over a 4-day culture period. One of these defined media, AIM V, supported LAK cell activation comparable to that of serum-containing medium for a 7-day culture period. Additionally, comparable activation in AIM V medium was observed when cells were cultured at a density of 2 × 10⁴ cells/ml with 600 units/ml IL-2 compared to 1 × 10⁴ cells/ml with 1000 units/ml IL-2 in serum-containing medium. Therefore, LAK cells can be generated in AIM V medium in half of the volume of medium and less IL-2. This clearly provides an advantage over the current dependence on human AB serum.

In sum, the current studies suggest that a number of minor modifications in the current procedures for generating LAK cells for cancer clinical trials should be considered in the design of future clinical protocols, i.e., culture for longer periods of time, in the presence of CO₂, and in defined media. However, it is still important to address basic questions regarding the mechanisms involved in LAK cell activation and the maintenance of such activity over longer periods of time, both in vitro and in vivo. A better understanding of the biochemical mechanisms of IL-2 stimulation of LAK cell differentiation may provide insight into methods to improve LAK cell generation and in vivo activity and better manage the toxicity of IL-2 associated with their clinical use.

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