Cancer Immunol Immunother (1989) 30: 1–4

Cancer
Immunology
Immunotherapy
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A new high-yield continuous cell-culture system for lymphokine-activated killer cells

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Summary. We performed basic studies on a new high-yield culture system (concentrate rotary tissue-culture system) for application to adoptive immunotherapy with lymphokine-activated killer (LAK) cells. Using this system, we demonstrated that up to 2×10^7 peripheral blood mononuclear cells/ml could be cultured in interleukin-2 with a sufficient recovery rate and cytotoxicity in short-term cultures (6 days). This system can also be used to proliferate LAK cells to four times the initial cell number with sufficient cytotoxicity for 14 days of culture. Thus, this system allows activation of sufficient numbers of cells to conduct clinical trials on humans.

Introduction

Recently, combined adoptive immunotherapy with interleukin-2 (IL-2) and lymphokine-activated killer (LAK) cells has proved to be effective in treating some cancer patients [1, 7, 9]. Since 1×10^{11} LAK cells are thought to be necessary in order to culture such a large amounts of cells, huge culture bottles or many regular bottles are usually necessary. However, it is very cumbersome to handle such a large number of bottles without contaminating them. A large amount of expensive IL-2 must also be used.

A new compact and cell-dense continuous culture system was recently developed, in which mouse hybridoma cells can be successfully cultured with a high yield of antibody and cells [6]. In this paper, we report the results of basic studies on this system to be used for adoptive immunotherapy with LAK cells.

Materials and methods

Cell preparations. For large-scale production of LAK cells using a new culture system, human peripheral blood mononuclear cells (PBMC) were obtained by lymphopheresis using a continuous cell separator (IBM-2997, Hopewell Junction, NY). About 5 l whole blood were processed at a flow rate of 50–60 ml/min over a period of 2–3 h. Acid citrate/dextrose-A (Terumo, Tokyo, Japan) was added to the collection bag at the time of lymphopheresis. The final volume in the lymphopheresis bag (Kawasumi Laboratories Inc., Kanagawa, Japan) was usually 300 ml. The bag was then centrifuged at 300 *g* for 15 min to remove platelets. This process was repeated three to five times. Finally, the cells were resuspended in Hanks' balanced salt solution (Gibco, Grand Island, NY) containing 1% human serum albumin. To isolate the mononuclear cells, the cells were layered on Ficoll/Conray (1.077 g/ml) made of Ficoll-400 (Pharmacia Fine Chemicals, Uppsala, Sweden) and Conray-400 (Daiichi, Tokyo, Japan) in a blood cell processor (IBM-2991, Hopewell Junction, NY) using a bag-to-bag system.

High-yield culture system (concentrate rotary tissue-culture system). The system consisted of a culture bag on a rotator (rotary agitator, KL-5000, Kawasumi Laboratories, Kanagawa, Japan). The culture bag (concentrate culture bag, CC-5100, Kawasumi Laboratories, Kanagawa, Japan) had two compartments, an inner compartment separated from the outer compartment by a semipermeable membrane (pore size: 2.4 nm) (Fig. 1). Cells in the appropriate culture medium were placed in the inner compartment (capacity: 1000 ml) and medium without serum was placed in the outer compartment (capacity: 4 l). The bag was rotated at an angle of 45° between 0.5 rpm and 5 rpm in a 37° C incubator (Fig. 2). A CO_2 incubator was not required because the concentration of cells was high and therefore sufficient CO_2 was produced by the cultured cells. The medium was changed in the outer compartment at intervals when needed, but no medium changes were needed in the inner part for short-term cultures. The outer compartment acted as the feeder as well as allowing catabolites to diffuse through the culture.

Cell cultures. Purified human recombinant IL-2 with a specific activity of 3.5×10^4 units/mg protein was kindly provided by Takeda Chemical Industries Ltd [3]. One Takeda unit of IL-2 activity was equivalent to 343 Biological Response Modifier Program units. Cells were cultured in an inner compartment of a culture bag with 500 ml RPMI 1640 medium, 20% heat-inactivated human pooled AB serum, various concentrations of recombinant IL-2, and the same volume of disinfected air. The outer bag contained 2 l RPMI 1640 and the same volume of air.

For some studies, PBMC were suspended at 2×10^6/ml in RPMI 1640 medium supplemented 20% human pooled AB serum. The cell suspensions were supplemented with various amounts of IL-2 and were incubated in 25-cm^2

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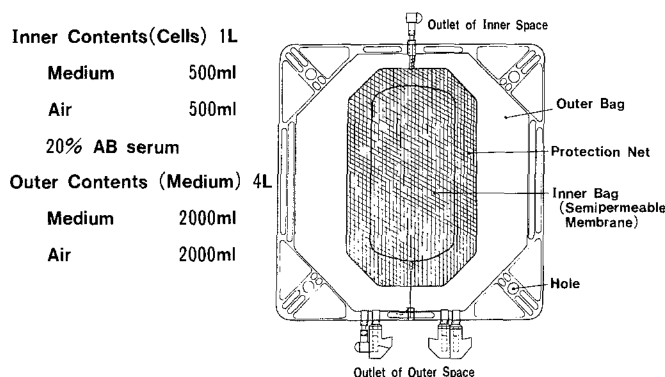

Fig. 1. Diagram of a culture bag

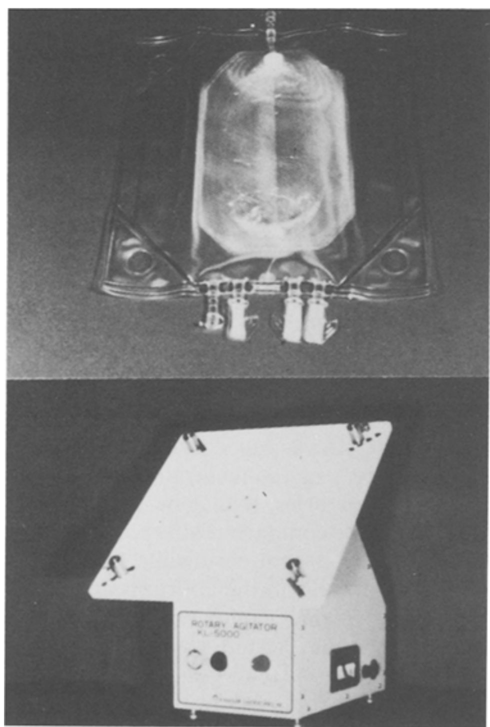

Fig. 2. New high-yield continuous cell-culture system, consisting of a culture bag on a rotator

tissue-culture flasks (Corning 25100) in a 5% CO_2, 95% air atmosphere at 37° C.

Cytotoxicity assay. The natural-killer (NK)-resistant human B cell lymphoma Raji cell line used as a target was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum. The medium was changed 1 day before use.

Cytotoxicity was measured by ATP assay [2]. Briefly, the assays were performed in RPMI 1640 medium supplemented with 10% fetal calf serum. Cytotoxicity was tested in U-bottomed wells of 96-well plates (Sumitomo Bakelite, Tokyo, Japan) with 200 µl assay medium containing 1×10^4 target cells and various numbers of effector cells. The assays were initiated by low-speed centrifugation (50 *g* for 4 min) followed by incubation at 37° C for 4 h. At the end of the incubation, cells were resuspended in 100 µl phosphate-buffered saline. The ATP levels were determined by a previously described technique [4]. Triton X-100 (Wako Pure Chemical Industries, Osaka, Japan) was added to each well and 30 µl supernatant was collected in microcuvettes for measurement of ATP. Then, 250 µl 0.01 M Hepes buffer (pH 7.75) was added to each tube followed by addition of 100 µl luciferin/luciferase, (FLE-50, Sigma, St. Louis, Mo). The luminescence was measured by a photometer (Chem-Glow, Aminco, Silver Spring, Md). Cytotoxicity was determined by the following formula.

$$\text{Cytotoxicity (\%)} = \frac{(B + C) - A}{C} \times 100$$

A, experimental ATP luminescence; *B*, ATP luminescence of effector cells alone; *C*, ATP luminescence of target cells alone. Each assay was performed in triplicate.

Results

Studies of culture conditions in the new culture system

To determine the proper conditions using the new culture system for the large-scale production of LAK cells with appropriate cytotoxicity, studies were performed using a miniaturized bag consisting of a 20-ml inner compartment and a 1000-ml outer compartment. PBMC were cultured in an inner bag with 10 ml RPMI 1640 medium, 20% human pooled AB serum and various amounts of IL-2. The outer compartment contained 500 ml of RPMI 1640 medium.

We first studied the effects of cell concentrations on LAK induction. PBMC from five healthy donors were cultured in 1.0 U/ml IL-2 for 6 days at various cell concentrations. As shown in Fig. 3, PBMC cultured at a concentration of 2×10^7/ml exhibited the greatest cytotoxic activity against Raji cells.

Next, to examine the effect of IL-2 concentrations on LAK induction in this culture system, PBMC from normal donors were cultured for 6 days in the presence of various amounts of IL-2. The results shown in Fig. 4 are from a representative experiment. LAK activity reached a plateau above 2 U/ml IL-2.

Short-term culture of PBMC using the new culture system

Short-term cultures of PBMC from six patients with various cancers were performed using this new culture system. A mean ± SD of $7.1 \pm 4.3 \times 10^9$ PBMC (range: 1.7×10^9 to 11.5×10^9) was obtained by one lymphopheresis. Whole cells, except for cells cultured in a tissue-culture flask in parallel experiments, were suspended in 500 ml RPMI 1640 medium supplemented with 20% human pooled AB serum and 2 U/ml IL-2 in the inner compartment of the bag and cultured for 6 days without changing the 2 l of culture medium in the outer compartment. For comparison, 2×10^7 cells were cultured for 6 days in a 25-cm^2 tissue-culture flask at 2×10^6 cells/ml with 20% human pooled AB serum and 0.5 U/ml IL-2, which was optimal for flask cultures (data not shown). A representative result of the kinetics is shown in Fig. 5. Although cell recovery from both culture systems appeared to be equivalent, cytotoxicity was higher in the bag system after a 6-day culture. On completion of the culture, a significantly higher cytotoxicity could be obtained in the bag system than in the flask ($P < 0.01$ by paired *t*-test) as shown in Fig. 6.

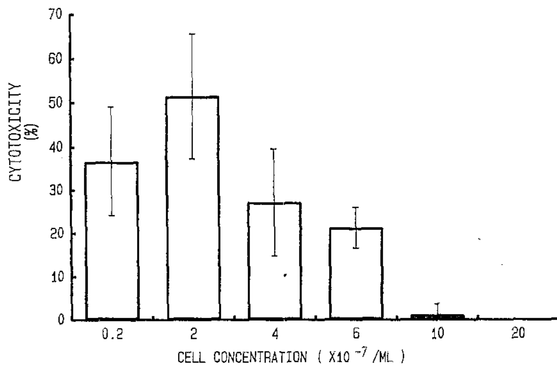

Fig. 3. Effect of cell concentrations on LAK induction. Peripheral blood mononuclear cells (PBMC) from nine healthy donors were cultured in 1.0 U/ml interleukin-2 (IL-2) for 6 days at various cell concentrations and then assayed for cytotoxicity against Raji cells. The data shown are at an E/T ratio of 20:1. The values represent the mean ± SD

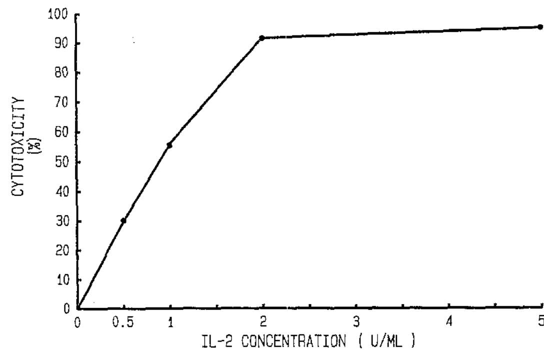

Fig. 4. Effect of IL-2 concentrations on LAK induction. PBMC were cultured for 6 days in the presence of various amounts of IL-2. The cytotoxicity data are shown at an E/T ratio of 20:1

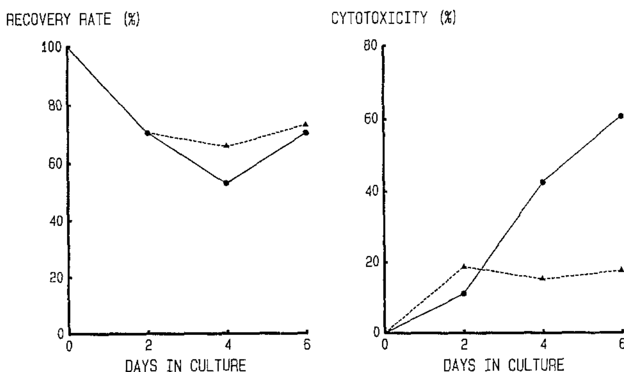

Fig. 5. Kinetics of cell numbers and cytotoxicity. PBMC were cultured using bags with 2×10^7 cells/ml and 2 U/ml IL-2 (●) and flasks with 2×10^6 cells/ml and 0.5 U/ml IL-2 (▲). Cell numbers were serially counted and expressed as percentages of the initial numbers. The killing kinetics for Raji cells are shown at an E/T ratio of 10/1

High-yield culture of PBMC using a new culture system

To obtain a large number of LAK cells, the culture period for proliferation of PBMC cultured with IL-2 in this system was extended. PBMC obtained by nine lymphophereses from five cancer patients were cultured in 5 U/ml IL-2 for 14 days. One half of the IL-2 containing medium of the inner compartment was changed every 4–5 days, while the medium in the outer compartment was totally changed every 3–4 days, usually when the color turned to yellow. The results are summarized in Table 1. The initial cell number obtained by lymphophereses was $(4.1 \pm 2.5) \times 10^9$ with a range of $(1.7–9.2) \times 10^9$. Although the recovered cell numbers varied from case to case, some reduction in numbers was noted until 7 days after initiation of the culture. Thereafter, the numbers increased to more than four times the initial cell numbers at the end of the culture, 14 days later. In one case, 13.2 times the initial cell number could be obtained after 2 weeks of culture. In terms of LAK activity against Raji cells in the cultured cells, the cytotoxicity increased from 40.3 ± 12.4% on day 4 to 71.9 ± 14.8% at the end of the culture.

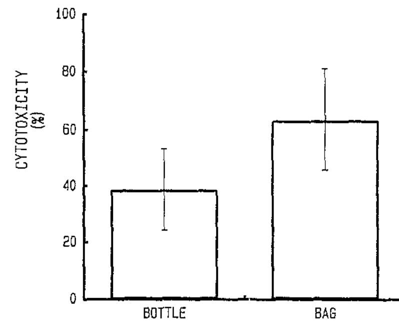

Fig. 6. Cytotoxicity of LAK generated in bags and flasks. PBMC from six cancer patients were cultured for 6 days in bags with 2×10^7 cells/ml and 2 U/ml IL-2 and in flasks with 2×10^6 cells/ml and 0.5 U/ml IL-2. Cytotoxicity of LAK cells generated in the bag system was significantly higher than that in a flask ($P < 0.01$ by a paired *t*-test). The data are shown at an E/T ratio of 10/1

Table 1. Results of 2-week cultures using a new culture system

Time in culture[a] (days)	Recovery rate (fold)		LAK cytotoxicity[b] (%)	
	Mean ± SD	Range	Mean ± SD	Range
4	0.69 ± 0.11	0.54– 0.88	40.3 ± 12.4	23.5–57.5
5	0.67 ± 0.10	0.57– 0.85	46.9 ± 13.9	27.5–72.3
6	0.64 ± 0.13	0.51– 0.87	51.6 ± 13.8	32.0–70.0
7	1.13 ± 0.81	0.52– 3.0	55.0 ± 14.7	36.0–73.5
8	1.67 ± 1.46	0.62– 5.1	59.4 ± 15.3	40.5–80.0
9	2.22 ± 2.08	0.69– 7.3	64.9 ± 17.2	44.5–89.0
10	2.82 ± 2.71	0.82– 9.5	69.5 ± 18.8	49.0–98.3
11	3.25 ± 2.84	1.09–10.4	71.3 ± 17.8	53.4–100
12	3.66 ± 3.02	1.23–11.3	72.9 ± 15.9	52.5–97.1
13	3.97 ± 3.25	1.36–12.3	70.3 ± 17.1	32.1–86.0
14	4.33 ± 3.44	1.50–13.2	71.9 ± 14.8	47.3–88.0

[a] PBMC obtained by nine lymphophereses from five cancer patients ranged from 1.7×10^9 to 9.2×10^9; mean ± SD: $(4.1 \pm 2.5) \times 10^9$. Cells were cultured in the inner compartment containing 500 ml RPMI 1640 medium supplemented with 5 U/ml IL-2 and 20% human pooled AB serum for 14 days. The inner contents were divided into two bags usually every 4–5 days by replacing half of the volume with a new medium including IL-2

[b] Cytotoxicity was assayed against Raji cells and the data are shown at an E/T ratio of 10/1

Discussion

For large-scale production of LAK cells, a 2.3-l roller bottle containing 1000 ml PBMC and culture medium sup-

plemented with IL-2 has been used in several clinical studies of adoptive immunotherapy with LAK cells [1, 7, 9] since Muul and her colleagues revealed its usefulness [5]. According to their study, 1.5×10^6 cells/ml is a proper cell concentration for such cultures. To culture 1×10^{10} PBMC with IL-2, as many as six or seven roller bottles would be required, and 6–7 l culture medium containing IL-2 would be necessary. Recently, gas-permeable culture bags have been also used to generate LAK cells for adoptive immunotherapy trials. Topalian and her colleagues [8] found that tumor-infiltrating lymphocytes could be expanded with IL-2 more rapidly in gas-permeable culture bags than in roller bottles. However, they split the cells at a concentration of $(2.0-3.0) \times 10^6$/ml, which seems to be a proper cell density for such cultures using gas-permeable culture bags.

In this study, we demonstrated how a cell concentration as high as 2×10^7/ml can be used to obtain sufficient LAK cytotoxicity using our new culture system. Therefore, 1×10^{10} PBMC can be cultured in 500 ml medium in the inner compartment and 2 l outer medium of a single bag. Only 500 ml IL-2-containing medium is needed. Since this is a closed-system, sterile conditions can easily be maintained. Another major advantage is that this system uses a regular 37° C incubator instead of a CO_2 incubator.

According to experimental studies in animal models, 10^{11} immune cells are required to treat clinically evident human cancers. To obtain such large numbers of activated cells, about five leukaphereses are usually performed [1, 7]. Therefore, it would be very useful if LAK cells could be made to proliferate with sufficient cytotoxicity. In this study, we demonstrated that our new culture bag can be used for the proliferation of PBMC cultured in IL-2 to four times the initial cell number in 14 days. These proliferating cells were found to have sufficient cytotoxicity for clinical use.

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Received 30 December 1988/Accepted 28 February 1989