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PROLIFERATION OF ANTI-CD_3 McAb AND IL-2 INDUCED SPLENOCYTES AND ANTITUMOR EFFECT OF THEIR CULTURE SUPERNATANTS*

Shen Guanxin 沈关心 Wang Xialin 王晓林 Zhu Huifen 朱慧芬
Zhang Yue 张悦 Shao Jingfang 邵静芳

Department of Immunology, Research Center of Experimental Medicine, Tongji Medical University, Wuhan 430030

The proliferation of splenocytes from health adults was induced by anti-CD_3 McAb and IL-2. The proliferative potential of the splenocytes and antitumor activity of their culture supernatants of splenocytes were studied. The results showed that anti-CD_3 McAb not only enhanced the proliferation of the splenocytes directly, but also enhanced that of induced by IL-2. Their enhancing effect was more significant when the incubation time *in vitro* was prolonged. The culture supernatants of anti-CD_3 and IL-2 induced splenocytes also had the antitumor activity and enhancing capability to the antitumor activity of LAK cells. The results suggested that LAK cells could secret lymphokine, and this effect would be synergically promoted when anti-CD_3 and IL-2 were simultaneously used.

Key words: Anti-CD_3 McAb, IL-2, Splenocytes, Cultured supernatants.

The discovery of lymphokine-activated killer (LAK) cells has brought great hope for the adoptive immunotherapy (AIT) of cancer. It was demonstrated that the culture supernatants of the LAK cells were effective in antitumor activities.[1] However, in pursuit of more effective, yet generally applicable nonspecific way for propagating tumor-reactive T cells in culture for use in AIT, the current study examined the use of an antibody to CD_3 as the primary stimuli for T cell proliferation.[4, 5] This report examines the growth characteristics, proliferation and cytotoxic capacity of the splenocytes induced by anti-CD_3 monoclonal antibody (McAb) and interleukin 2 (IL-2) as well as the cytotoxic capacity of their culture supernatants *in vitro*. Those of IL-2 stimulated splenocytes and anti-CD_3, IL-2 co-stimulated splenocytes were compared in our research.

MATERIALS AND METHODS

Reagents

1. Recombinant human interleukin 2 (rhIL-2) purchased from Shanghai Institute of Cell Biology, Chinese Academy; 2. Anti-CD_3 monoclonal antibody produced by mouse hybridomas (gifted from Institute of Immunology, Essen University, FRG). McAb were purified by n-octoic acid precipitation method in our laboratory;[2] 3. MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyl tetrazolium bromide, Fluka); 4. Acidified

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isopropylalcohol (0.04 mol/L HCl isopropanol, Merck); 5. Costar plates purchased from USA; 6. ELISA-detector, product of Factory of Electronic Tube, East-China.

Culture of Leukemic Cell Line

Raji cells: a cell line of B-lymphocytic leukemia which is NK resistant and LAK-sensitive, were cultured in complete culture medium containing RPMI-1640 solution and 20% FCS. At the phase of logarithmic growth, the cells were collected and washed by incomplete culture medium. The cell viability (trypan blue dye exclusion) was always >95%. The concentration of the cells was adjusted into 2×10^5/ml by dilution with 20% FCS-RPMI 1640.

Passage and Culture of Splenocytes in Different Culture Medium Systems

The mononuclear cells (MNC) were isolated from the spleen of healthy adults. Briefly, the spleen was cut minced and mashed on a metal net. Cell suspension was then centrifugated on FicollHypaque (d=1.007+0.001) at 2000 rpm for 20 min, the MNC-rich layer was harvested. After washed two times, the MNC suspension was diluted to 1×10^6 cell/ml with RPMI-1640 contained 20% FCS, the MNC suspension was supplemented by rhIL-2 or anti-CD_3 McAb combined with rhIL-2. The concentrations of rhIL-2 and anti-CD_3 McAb were 500 U/ml and 5 μg/ml, respectively. The above different isolated splenocytes were cultured at 37°C 5% CO_2 and passaged every 3–5 days. After incubation for 8 days, the cells and culture supernatants were collected.

Definition of Splenocyte Proliferation *in vitro*

After incubation for 3, 5 and 8 days, the number and proliferative rate of the splenocytes induced by different culture medium systems were examined and determined.

Detection of the Activity of IL-2 in Culture Supernatants of Splenocytes

The activity of IL-2 in culture supernatants of the splenocytes was determined by MTT colorimetry.[3] The cultured supernatants of the splenocytes and standard human recombination IL-2 (HrIL-2) were serially diluted in two fold, and poured into every well in costar plates (100 μl/well), then incubated with 2.5×10^5/50μl CTLL cell at 37°C, 5% CO_2 for 24 h. After the supernatant was replaced by 50 μl 500 μg/ml MTT 1640 solution, the cell were cultured for 4 h and treated by 50 μl/well acidified isopropylalcohol, then dispersed by agitation. The OD value of the suspend was detected by ELISA-detector at 570 nm, referring to that of standard HrIL-2, the activity of IL-2 in the supernatants of LAK cells was analyzed.

The Determination of Antitumor Activity of Culture Supernatants of Splenocytes

The culture supernatants of the splenocytes were adjusted into various concentrations with the same solution. MTT colorimetry was employed to evaluate the cytotoxic effect of the culture supernatants of the splenocytes on leukemic cells *in vitro* as following steps: The mixture of the culture supernatants and target cells was triplicately added into the wells of costar plates. The target cells were cultured lonely to determine the absolute absorbance of the controls of target cells. After incubation at 37°C for 20 h, and some steps which were similar to those described in the evaluation of IL-2 activity. Percentage cytotoxicity was calculated relative to the calibration standard target cell curve as follows:

$$\text{cytotoxicity \%} = \left(1-\frac{\text{ODs}}{\text{OD1640}}\right)\times 100\ \%$$

ODs=OD value of culture supernatant of splenocyte effector

OD1640=OD value of the controls of RPMI-1640

Detection of Enhancing Effects of Culture Supernatants on Antitumor Activity of LAK

The concentration of LAK cells was adjusted into 5×10^5/ml with above culture supernatants in various concentrations (0%, 50%, 100%). MTT colorimetry was employed to evaluate the cytotoxic effect of the LAK cells on leukemic cells *in vitro*: The mixture of

effector cells and target cells was triplicately added into the wells of costar plates. The effector cells in various concentrations were cultured lonely to determine the absolute absorbance. Similarly, the controls of the target cells were set. After incubation at 37°C for 20 h, and some steps which were similar to those described in the evaluation of IL-2 activity. Percentage cytotoxicity was calculated relative to the calibration standard target cells curve as follows:

$$\text{cytotoxicity } \% = \left(1 - \frac{OD^{E+T} - OD^{E}}{OD^{T}}\right) \times 100\ \%$$

OD^{E+T} =OD value of effector cells+OD value of target cell
OD^{E} =OD value of the controls of effector cells
OD^{T} =OD value of the control of target cells

RESULTS

The rhIL-2-induced Splenocyte Proliferation Modulated by Anti-CD_3 McAb

Table 1 showed that the number of the splenocytes *in vitro* was increased to approximately 2.8-fold after rhIL-2 for 8 days stimulation. Anti-CD_3 McAb enhanced the proliferation of the splenocytes induced by rhIL-2. During cultured in anti-CD_3 McAb combined with rhIL-2 for a period of 8 days, the splenocytes were increased to 6.2-fold, their proliferative effect was double as that induced by rhIL-2 alone.

Activity of IL-2 in the Culture Supernatants of the Splenocytes

Based on OD values of the supernatants of the splenocytes, the activity of IL-2 was read in the standard curve of HrIL-2 (Figure 1). The mean IL-2 activity in the supernatants of the splenocytes was 110.6 U/ml.

Cytotoxic Activity of the Culture Supernatants of the Splenocytes Induced by Anti-CD_3 and IL-2

As shown in Figure 2, cytotoxicity of the culture supernatants of the splenocytes induced by anti-CD_3 McAb+rhIL-2 on Raji cells was higher than that induced by IL-2 alone.

Table 1. rhIL-2 and anti-CD_3 McAb-induced splenocyte proliferation

	Splenocyte number		Proliferative rate
	Before culture	After culture	
rhIL-2	1×10^6	2.8×10^6	2.8 fold
rhIL-2+anti-CD_3	1×10^6	6.2×10^6	6.2 fold

Effects on Cytotoxic Activity of LAK by Culture Supernatants of Splenocytes

Figure 3 showed that the culture supernatants of anti-CD_3 and IL-2 induced splenocytes had the enhancing capability to promote antitumor activity of LAK cells. Their enhancing effect was more significant when the concentrations of the culture supernatants was increased. The enhancing effects for the culture supernatants of the splenocytes induced by was higher than that induced by IL-2 alone.

DISCUSSION

It has been generally believed that LAK precursor cells could be derived from the spleen. The spleen is biggest immune organ in human body and contains a great number of lymphocytes. The MNCs of the spleen

were confirmed as splenic LAK cells, which showed rapid proliferation and antitumor activity *in vitro* after rhIL-2 induction.[4, 5] Anti-CD_3 monoclonal antibody has enhancing capability to promote antitumor activity of IL-2 induced splenocytes.[6, 7] The results in this study suggested that anti-CD_3 McAb enhanced significantly the proliferation of the splenocytes induced by rhIL-2, moreover. Their enhancing effects were more significant when the incubation time *in vitro* was prolonged (Figure 1). Anti-CD_3 McAb induces T cell activation and proliferation via the TCR/CD_3 complex, and enhances expression of IL-2 receptor on T lymphocytes.[6, 8] The stimulation of the TCR/CD_3

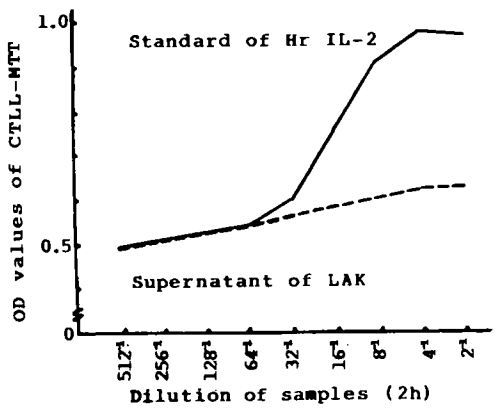

Fig. 1. Activity of IL-2 of HrIL-2 standard, cultured supernatant of LAK cells.

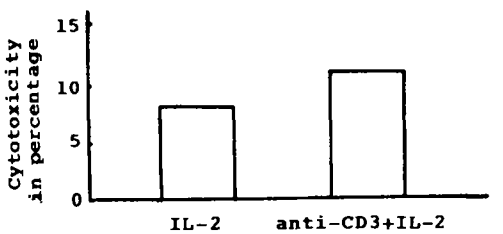

Fig. 2 Cytotoxic activity of the culture supernatants of the anti-CD_3 and IL-2 induced splenocytes.

complex with anti-CD_3 McAb has been shown to provide a means of mimicking the normal pathways of T cell activation. Our results showed that anti-CD_3 could induce proliferation of the splenocytes, moreover activated and proliferated lymphocytes could secret great number of lymphokine, which can enhance IL-2-induced LAK proliferation. Figure 1 demonstrated that proliferative effects of the splenocytes induced by anti-CD_3 McAb+rhIL-2 were greater than that by rhIL-2 alone, it was almost as double as that by rhIL-2 alone. This result suggested that anti-CD_3 McAb indirectly promoted antitumor activity of LAK cells by enhancing splenocyte proliferation.

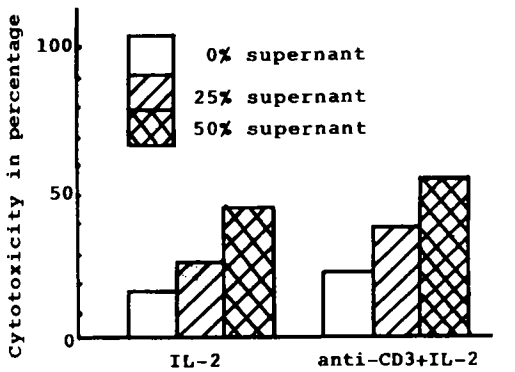

Fig. 3. Effects on cytotoxic activity of LAK by culture supernatants of splenocyte

This study demonstrated that the culture supernatants of anti-CD_3 and IL-2 induced splenocytes had the antitumor activity and capability to promote antitumor activity of LAK cells. As shown in Figure 3, cytotoxicity of the culture supernatants of anti-CD_3 McAb+rhIL-2 induced splenocytes on Raji cells was higher than that of IL-2 induced splenocytes. This results suggested that anti-CD_3 McAb can enhance antitumor activity of the culture supernatants of IL-2 induced splenocytes.

This experiment demonstrated that the culture supernatants of anti-CD_3 and IL-2 induced splenocytes had the capability to promote antitumor activity of LAK cells. Their enhancing effect was more significant when the concentrations of the culture supernatants was increased. The enhancing effects of the culture supernatants of anti-CD_3 McAb+rhIL-2 induced splenocytes on cytotoxicity of LAK cells was higher

than that of IL-2 induced splenocytes. The LAK mediated cytotoxicity on Raji cells was elevated continuously (90%) when the ratio of effector cells and target cells in 5:1, and the concentration of the culture supernatants of anti-CD_3 and IL-2 induced splenocytes in 50%.

The results showed that the culture supernatants of anti-CD_3 and IL-2 induced splenocytes not only directly killed tumor cells, nut also indirectly killed tumor cells through activated and enhanced LAK cells. Antitumor activity of the culture supernatants of the splenocytes might be relevant to the lymphokine secreted by activated splenocytes. This results showed that the activity of IL-2 in the supernatants of the splenocytes was so high that it could support the growth of IL-2 dependenting CTLL cells or other cells, promote LAK mediated cytotoxicity on malignant cells and gratify the need of investigating its clinical application even though it was diluted at 1:16 to 1:32.

The mechanism of LAK cells on killing tumor cells might be that LAK cells could directly bind to tumor cells, and the activated and proliferated lymphocytes induced by anti-CD_3 McAb and IL-2 could secret great number of lymphokine, such as tumor necrosis factor (TNF), interferon (IFN) and IL-2. This study demonstrated that anti-CD_3 can enhance antitumor activity of LAK cells through secreted lymphokine of IL-2-activated LAK. Synergism of enhancing effects of anti-CD_3 and IL-2 on antitumor activity of LAK cells will be signifcant to guide the cellular adoptive immunotherapy on patients with malignant tumors, and preparation of tumor infiltrating lymphocytes (TIL).

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