0014-4754/93/010076-04$1.50 + 0.20

Induction of lymphokine-activated killer cells from nude mouse spleen cells by interleukin-4

I. S. Tunru, H. Suzuki and M. Kobayashi

First Department of Internal Medicine, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-01 (Japan)
Received 16 July 1992; accepted 16 October 1992

Abstract. The induction of lymphokine-activated killer (LAK) cells from natural killer (NK) lineage cells by interleukin-4 (IL-4) was studied in vitro. Activation of nude mouse spleen cells by IL-4 generated cytotoxic cells, capable of killing NK-sensitive as well as NK-resistant tumor cells. The induction of peak lytic activity was demonstrated after 3 days of culture with IL-4. Surface marker analysis indicated that the majority of precursor cells were aGM1$^+$, Thy1$^-$, and the majority of effector cells were aGM1$^+$, Thy1$^+$, suggesting that IL-4 induced LAK cells from nude mouse spleen cells were similar to those from normal mouse spleen cells. The induction of nude mouse LAK cells by IL-4 was partially inhibited by anti-IL-4 or anti-interferon (IFN)-α,β antibody, and it was further inhibited by the combination of two antibodies, suggesting that IFN-α,β production was associated with LAK induction of NK lineage cells by IL-4.
Key words. IL-4; LAK cells; nude mouse.

Activation of human as well as murine lymphocytes with interleukin-2 (IL-2) induces cytotoxic cells named lymphokine-activated killer (LAK) cells, which lyse a variety of tumor cells[1]. LAK cells and their precursors are heterogenous, and LAK effectors consist of at least two distinct populations, namely natural killer (NK) LAK (NK-LAK) and T cell LAK (T-LAK) cells[2]. The NK-LAK cells display anti-tumor cytotoxicity similarly to T-LAK cells, although they show preferential activity against NK-sensitive tumor cells[2]. The direct induction of NK-LAK cells by IL-2 from NK lineage cells was demonstrated in nude mice which lack cytotoxic T cell precursors[3,4]. LAK cells generated from Thy1-negative nude spleen cells were shown to express Thy1 antigen, but not CD3 molecules. They were more resistant to the treatment with anti-asialo GM1 (aGM1) antibody plus complement than normal nude mouse spleen cells with NK activity.

In addition to the IL-2 induced LAK phenomenon, recent studies have demonstrated that IL-4, initially termed B-cell stimulatory factor-1, can also induce LAK activity[5–7]. It was demonstrated that this cytokine could induce LAK activity with a wide spectrum of activity against tumor cells similar to that of IL-2-induced LAK cells; however, IL-4 generated more T-LAK cells than NK-LAK cells compared to IL-2[5]. Therefore, it seemed interesting to investigate whether IL-4 could induce LAK cells directly from NK lineage cells. In this study, we examined the generation of LAK cells by IL-4 from young nude mouse spleen cells, which lack T lineage cells. The precursor and effector phenotypes were investigated.

Materials and methods

Mice. BALB/c *nu/+* and *nu/nu* mice were purchased from Shizuoka Laboratory Animal Center, Hamamatsu, Japan. BALB/c *nu/nu* mice bred in our animal facility were used at ages of 4–7 weeks.

Media. Cytotoxic assays were carried out in RPMI 1640 medium containing 10% heat-inactivated FCS. RPMI 1640 supplemented with 2 mM L-glutamine, 12.5 mM HEPES, 0.05 mM 2-mercaptoethanol, 100 U/ml penicillin, 30 μg/ml gentamicin, and 10% heat-inactivated FCS was used for cell cultures.

Reagents. Murine recombinant IL-4 was supplied by Ono Pharmaceutical Co., Tokyo, Japan. IL-4 activity was determined by the supplier. Anti-aGM1 heteroserum was obtained from Wako Pure Chemical Industries Ltd., Osaka, Japan. Anti-mouse IFN-α,β antibody was obtained from Pasel + Lorei, GmbH and Co., Frankfurt. The monoclonal antibody producing hybridomas, anti-IL-4 (11B11) and anti-Thy1.2 (J1j), were obtained from the American Type Culture Collection (ATCC, Rockville, MD), and culture supernatants of these hybridomas were used in this study.

Tumor cell lines. YAC-1 and P815 tumor cells were maintained in vitro as cell lines. YAC-1 is a Moloney virus-induced lymphoma of A/Sn origin and P815 is a mastocytoma of DBA origin. These lines were used as NK-sensitive and NK-resistant target cells, respectively.

Generation of LAK cells. BALB/c *nu/nu* spleen cells were cultured at 3×10^6 cells/ml with various concentrations of IL-4 in 24-well macroplates at 37 °C in 5% CO_2. After 3 days' incubation, cells were collected, separated by centrifugation on a Ficoll-Hypaque gradi-

ent and used as effectors in a 4-h ^{51}Cr-release assay. In some experiments, anti-IL-4 (1:10 dilution of hybridoma supernatant) or anti-IFN-α,β antibody (100 IU inhibitable) was added to LAK generation cultures.

Antibody and complement treatment of precursor or effector populations. Precursor populations (normal spleen cells) and effector populations of spleen cells (activated in vitro for 3 days with IL-4) were adjusted at 5×10^6 cells/ml and 2×10^6 cells/ml, respectively, and were treated with anti-Thy1 plus complement, anti-aGM1 plus complement, or complement alone for 50 min at 37 °C. Hybridoma supernatant of anti-Thy1 was used at a 1:8 dilution and anti-aGM1 was used at a 1:50 dilution. Rabbit complement (Low Tox-M rabbit complement, Cederlane Labo.) was used at a 1:8 dilution. The cells were washed twice with RPMI 1640, and then treated precursor populations were cultured with 300 U/ml IL-4 for 3 days, and effector populations were assayed immediately for cytolytic activity.

Cytotoxic assays. Target cells (5×10^5) were labeled with 100 µCi ^{51}Cr during 1-h of incubation at 37 °C in 5% CO_2. Effector cells in 80 µl medium were serially diluted in a V-bottom 96-well microtiter plate (Costar, Cambridge, MA) and mixed with 5×10^3 ^{51}Cr-labeled target cells in 80 µl medium. The plates were spun at $650 \times g$ for 3 min. After a 4-h incubation, 80 µl of each supernatant were measured for radioactivity with a gamma counter. The percentage of specific ^{51}Cr release was calculated using the following formula:

$$\text{Specific lysis} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximal release} - \text{spontaneous release}} \times 100$$

Experimental release was the average of release in duplicate cultures, spontaneous release was determined by incubating targets in assay medium, and maximal release was determined by incubating in detergent. The spontaneous release was always <20% of the maximum release. One lytic unit (LU) was defined as the number of effector cells mediating 30% specific lysis of 5×10^3 target cells, and was determined from the dose-response curve.

Results

To investigate whether IL-4 could induce LAK activity from NK-lineage cells, splenic lymphocytes of young nude mice were cultured with various concentrations of IL-4 for 3 days, and they were tested for lytic activities against NK-sensitive YAC-1 and NK-resistant P815 tumor cells. As shown in figure 1, nude mouse spleen cells cultured with IL-4 for 3 days showed cytotoxic activities against both YAC-1 and P815 tumor cells. The induction of cytotoxicity was dependent on the concentration of IL-4, and the optimal concentration of IL-4 for induction of cytotoxicity was 300 U/ml.

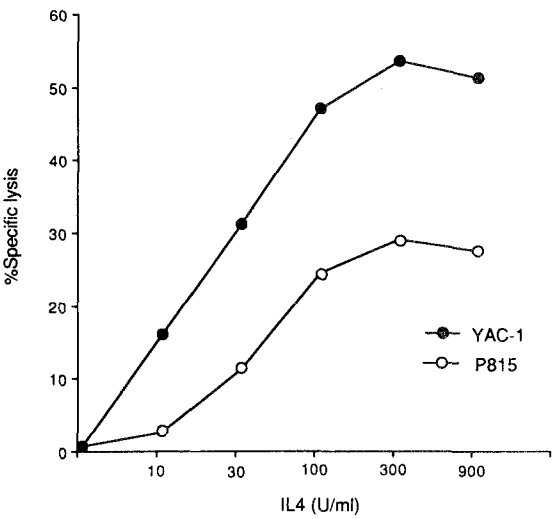

Figure 1. Induction of nude mouse LAK activity by IL-4. BALB/c nude mouse spleen cells were cultured at 3×10^6 cells/ml with various concentration of IL-4 for 3 days and tested for their cytotoxic activities against YAC-1 and P815 tumor cells at an effector to target ratio of 40:1.

The kinetics of LAK induction by IL-4 were then studied. Spleen cells were cultured for various periods with 300 U/ml IL-4, then were assayed for LAK activity. As shown in figure 2, IL-4 induced peak lytic activities against both YAC-1 and P815 tumor cells at 3 days of culture. These lytic activities rapidly decreased after 7 days of culture.

To examine the surface markers of precursor populations, spleen cells were treated with anti-Thy1 plus complement or anti-aGM1 plus complement before culturing with IL-4. Depletion of aGM1-bearing cells before culture significantly eliminated the generation of

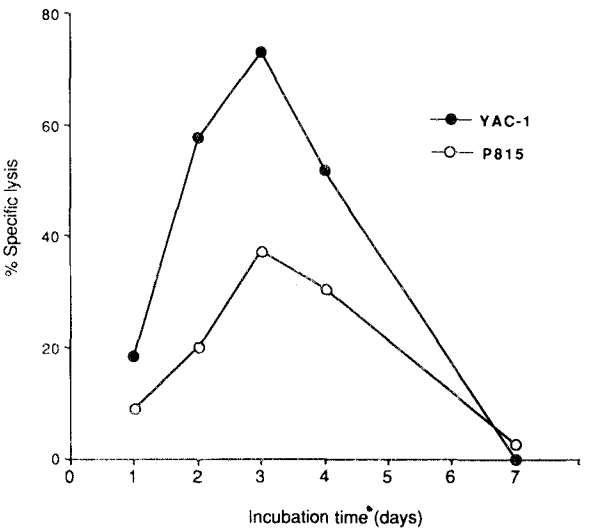

Figure 2. Kinetics of nude mouse LAK activity induction by IL-4. Spleen cells were cultured at 3×10^6 cells/ml with 300 U/ml IL-4 for 3 days and assessed for their cytotoxic activities against YAC-1 and P815 tumor cells at an effector to target ratio of 40:1.

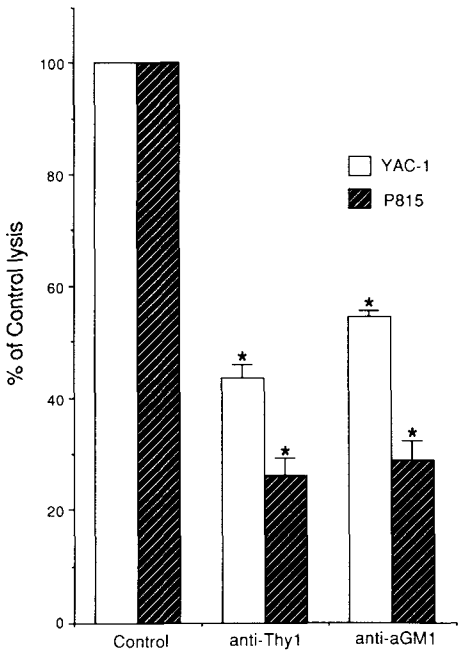

Figure 3. Surface markers of precursor populations. Normal nude mouse spleen cells were adjusted to 5×10^6 cells/ml, and were treated with anti-Thy1 plus complement, anti-aGM1 plus complement, or complement alone. Surviving cells were then cultured with 300 U/ml IL-4 for 3 days and assessed for their cytotoxic activities against YAC-1 and P815 tumor cells at an effector to target ratio of 50:1. Results indicate mean ± SD from percent of control lysis values obtained from three experiments. Statistical significance of the difference was analyzed by Student's t-test (*$p < 0.01$).

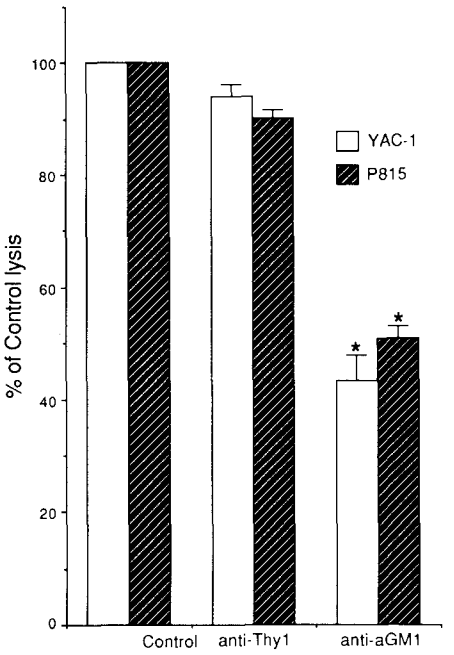

Figure 4. Surface markers of effector populations. Nude mouse spleen cells cultured with 300 U/ml IL-4 for 3 days were treated with anti-aGM1 plus complement, anti-Thy1 plus complement, or complement alone, and remaining cells were assessed for their cytotoxic activities against YAC-1 and P815 tumor cells at an effector to target ratio of 50:1. Results indicate mean ± SD from percent of control lysis values obtained from three experiments. Statistical analysis was performed by Student's t-test (*$p < 0.01$).

IL-4 induced LAK activity against YAC-1 and P185 tumor cells (fig. 3). On the other hand, elimination of Thy1$^+$ cells before culture failed to eliminate IL-4-induced LAK activity. Thus, the majority of IL-4 induced LAK cell precursors of nude mice are aGM1$^+$, Thy1$^-$. The surface markers of effector populations were studied next. After activation with IL-4, nude mouse spleen cells were treated with anti-aGM1 plus complement or anti-Thy1 plus complement, and remaining cells were assessed for their lytic activities. As shown in figure 4, depletion of both Thy1$^+$ and aGM1$^+$ cells in effector populations resulted in a significant reduction of cytotoxicity against YAC-1 and P815 tumor cells. Thus, the majority of IL-4 induced LAK cell effectors of nude mice are aGM1$^+$, Thy1$^+$.

Since IFN was reported to be produced when mouse spleen cells were stimulated with IL-2[8,9], the involvement of IFN(s) in the induction of LAK activity by IL-4 was then studied. Nude mouse spleen cells were cultured with 100 U/ml IL-4 in the presence or absence of anti-IL-4 and/or anti-IFN-α,β antibodies for 3 days, and their cytotoxic activity against YAC-1 tumor cells was assessed. As shown in figure 5, the induction of LAK activity by IL-4 was inhibited by either anti-IL-4 or anti-IFN-α,β antibody, and further inhibited by the combination of two antibodies, suggesting that IFN(s)

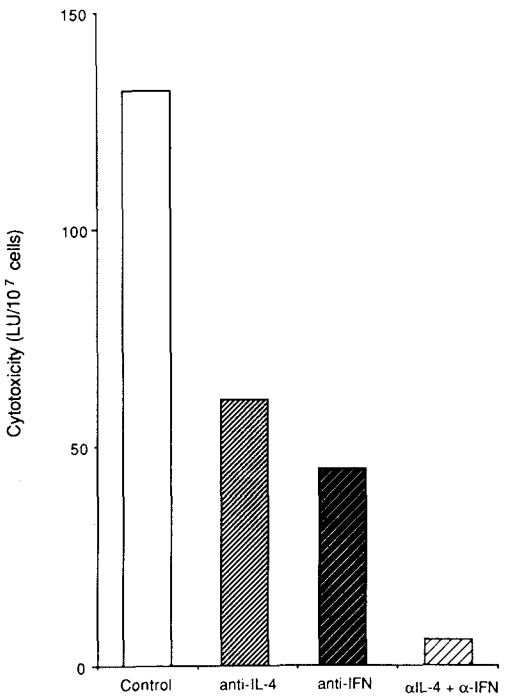

Figure 5. Inhibitory effects of anti-IL-4 and anti-IFN-α,β antibodies on the induction of nude mouse LAK activity by IL-4. Spleen cells were cultured with 100 U/ml IL-4 in the presence of anti-IL-4 (1:10 dilution of hybridoma supernatant) and/or anti-IFN-α,β (100 IU inhibitable) antibodies for 3 days and assessed for their lytic activity against YAC-1 tumor cells. Cytotoxicity was expressed as LU30%/10^7 effectors.

produced in culture was involved in IL-4 induced LAK activity in nude mice.

Discussion

The present study has demonstrated that IL-4 can induce LAK cells directly from NK lineage mouse lymphocytes. To examine LAK cell induction from NK lineage cells, we used nude mice less than 7 weeks old, because young (1- to 2-month-old) nude mice were shown to have undetectable levels of Thy 1 bearing cells in the spleen[10]. In this study, it was demonstrated that IL-4-induced nude mouse LAK cells showed lytic activities against both NK-sensitive and NK-resistant tumor cells. The majority of precursors and effectors of these killer cells were aGM1$^+$, Thy1$^-$ and aGM1$^+$, Thy1$^+$, respectively. Target specificities and surface markers of precursors and effectors shown in this study are similar to those of IL-4-induced LAK cells reported by us[7] and others[5,6] in normal mouse spleen cells. On the other hand, Nishimura et al.[3] have reported that LAK cells induced by IL-2 from nude mouse spleen cells are distinct from those induced from normal mouse spleen cells. They demonstrated that IL-2 induced LAK cells capable of killing a variety of tumor cells, from aGM1 , Thy1$^-$ precursors, and the generated effectors expressed Thy1, but not aGM1. They therefore suggested that IL-2 induced nude mouse LAK cells were distinct from aGM1$^+$, Thy1$^+$ LAK cells induced from normal spleen cells. The aGM1 marker is widely accepted as the marker of NK cells. In initial studies, it has been shown that anti-aGM1 treatment selectively eliminates NK activity in vivo and in vitro, whereas it does not affect cytotoxic T cells[11,12]. However, later studies have demonstrated that aGM1 is also expressed on T cells, monocytes, and polymorphonuclear leukocytes[13,14]. In addition this molecule is shown to be associated with certain cytotoxic T cell precursors and effectors[15]. Similarly, conflicting results have been reported on the expression of LAK effectors[16–18]. Some authors reported that LAK cells were aGM1-negative, others reported that they expressed aGM1, while the progenitors were shown to be almost eliminated by depletion of aGM1-expressing cells. Thus, it is suggested that the specificity of aGM1 varies with the detection assay and with the concentration of antiserum employed.

Earlier studies have demonstrated that murine NK activity is enhanced by IFN(s)[19]. In addition, it was shown that the induction of LAK cells by IL-2 was modified by IFN(s). Chao et al.[20] demonstrated the inhibition of murine LAK activity by IFN-γ, while Itoh et al.[21] suggested that human LAK cell induction involves a collaboration between IL-2 and IFN-γ. Stimulation of murine NK cells or lymphocytes with IL-2 was shown to produce IFN-γ, or -α,β[22]. In addition, we demonstrated recently that the induction of LAK activity by *Corynebacterium parvum* is dependent on endogenous IL-2 and IFN-α,β production[23]. These findings led us to investigate the possible involvement of endogenous IFN(s) in IL-4-induced LAK activity of NK lineage cells. The results demonstrated that the induction of nude mouse LAK cells by IL-4 was partially inhibited by anti-IFN-α,β antibody, suggesting that IFN-α,β production is associated with LAK cell induction of NK lineage cells by IL-4. However, it remains to be determined whether IL-4 stimulates NK cells to produce IFN(s), and the production of IFN(s) is required for LAK cell induction by IL-4.

Acknowledgment. We thank Akimi Takashima for her secretarial assistance in the preparation of this manuscript.

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