Responsiveness of Peripheral Blood Lymphocytes from Cancer Patients and Healthy Donors to Interleukin-2 (IL-2)

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KAMBE, M., MITACHI, Y., KANAMARU, R. and WAKUI, A. *Responsiveness of Peripheral Blood Lymphocytes from Cancer Patients and Healthy Donors to Interleukin-2 (IL-2)*. Tohoku J. exp. Med., 1988, **154** (2), 101-110 — The proliferative response of the peripheral mononuclear cells (MNC) from cancer patients and healthy individuals to IL-2 was studied by use of the quantitative microwell assay of the ^3H -TdR incorporation into the cells in vitro. After the addition of phytohemagglutinin (PHA), MNC acquired the reactivity to IL-2 within 3 hr, and reached a maximum after 12 to 17 hr of incubation. Although the IL-2 response of PHA-activated MNC from cancer patient was lower than that from healthy donor, there was no significant difference in the kinetics of proliferation. The maximum response of PHA-activated MNC from both cancer patients and healthy donors to IL-2 was observed at 96 hr and 84 hr, respectively. When monocytes were removed from MNC, IL-2-associated growth of the remaining fraction of MNC was decreased to 40-70% in both cancer patients and healthy donors. Furthermore, monocytes from cancer patients did not affect the IL-2 responsiveness of lymphocytes from healthy donors, and vice versa. The number of T lymphocytes having IL-2 receptors (IL-2R) from cancer patients was lower than that from healthy donors. These facts indicated that the lower IL-2 response of MNC from cancer bearer was due to the decreased number of T lymphocytes possessing IL-2R, and not due to monocytes themselves. ——— interleukin-2; interleukin-2 receptor; peripheral lymphocytes; cancer patients

In addition to antigen or lectin, the proliferation of T lymphocytes requires a lymphokine, T cell growth factor (TCGF) or Interleukin-2 (IL-2). A number of studies have shown that T lymphocytes express IL-2R on cell surface during stimulation with antigen or lectin, and the binding of IL-2 to these receptors drives T lymphocytes to proliferation and differentiation (Morgan et al. 1976; Gillis and Smith 1977; Larsson and Coutinho 1979; Smith 1980; Larsson 1981; Farrar et al. 1982). Immunological specificity of T cell clone is guaranteed by antigen-dependent activation, but not by lectin stimulation. IL-2 itself acts as nonspecific manner. Recently, much attention is paid whether IL-2 stimulates

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lymphocytes to expand in cancer patients, and gives an ability to escape from cancer. However, only a few about the lymphocyte-response to IL-2 in cancer patients were known. The present experiment was designed to see if there is any difference in the proliferative response of lymphocytes to IL-2 from cancer patients and from healthy donors.

MATERIALS AND METHODS

Patients. Twenty patients with advanced cancer, including 8 gastric, 4 large bowel, 3 pancreatic, 2 liver and 3 breast cancers, were entered in this study, and all cases were in the stage IV with the performance status of 0 to 2. The age of the patients ranged from 34 to 69, and all cases have experienced no premedication. As the control, 9 healthy males and 6 females with age ranging from 30 to 63 were entered in this study.

Reagents and IL-2. RPMI 1640 medium (Nissui Seiyaku Co., Ltd., Tokyo) supplemented with 5×10^{-5} M 2-mercaptoethanol (2-ME), 2.5×10^{-2} M HEPES buffer (Wako Junyaku Chemical Co., Ltd., Tokyo), 100 U/ml penicillin, 100 μ g/ml streptomycin (GIBCO Laboratories, Grand Island, NY, USA), and 10% (W/V) heat-inactivated (56°C, 30 min) fetal bovine serum (FBS, GIBCO Laboratories) were used for the culture, and the washing procedure.

IL-2 was obtained from Cell Science Laboratory (Electro-Nucleonics, Inc., Silver Spring, MD, USA), and a concentration of 50 U/ml, or 1:20, was used in the culture medium for maximal growth of MNC in these experiments.

Fractionation of MNC. Peripheral blood MNC were isolated by Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ, USA) density gradient centrifugation of heparinized blood (Böyum 1968). Separation of monocytes was performed by seeding MNC on plastic Petri dishes at 37°C in a humidified atmosphere containing 5% CO₂ in air for 3 hr. Nonadherent cells were removed by gently washing with PBS. These cells were peroxidase positive over 80% (Mosier 1967; Tan and Gordon 1971). Lymphocytes were separated from MNC by passing through Sephadex G-10 columns to remove adherent cells (Ly and Mischell 1974). More than 95% of these cells were lymphocytes as judged by morphology and peroxidase staining.

To obtain T lymphocytes, MNC were passed through nylon fiber (Wako Pure Chemical Ind., Ltd., Osaka) columns. More than 98% of the passing cells were T cells as judged by staining with phycoerythrin (PE)-conjugated monoclonal anti-human T3 antibody (Coulter Immunology, Hialeah, FL, USA).

Microassay of cell proliferation. Cells were stimulated with PHA (15 μ g/ml: Wellcome Reagents, Ltd., Beckman, England) at 37°C for 12 hr, at a final concentration of 1×10^6 cells/ml, washed three times to remove PHA, and suspended in the culture medium. One tenth ml of the cell suspension was seeded into microtiter wells (NUNCCLON®; Inter Med., Nunc, Denmark) and 0.1 ml of IL-2 at a final dilution of 1:20 was added. After desired hours of incubation at 37°C in 5% CO₂, ³H-thymidine (³H-TdR, 5 μ Ci/well, specific activity of 5 Ci/mM: New England Nuclear Corp., Boston, MA, USA) was added to each well, and incubation was continued for additional 4 hr. Cells in the wells were then harvested by multiple cell harvester (Labo Science Co., Ltd., Tokyo), trapped on glass fiber filters, and the radioactivity was counted with a liquid scintillation spectrometer (Beckman, Irvine, CA, USA) (Gillis et al. 1978; Schroff et al. 1983).

Absorption of IL-2. Various numbers of lymphocytes stimulated with PHA were sedimented by centrifugation, and 0.2 ml of diluted IL-2 was added. Absorption was performed at 37°C for 24 hr, with occasional shaking, and terminated by centrifugation of the cells at 600×g for 10 min. The remaining IL-2 activity in the supernatants was determined by the quantitative microwell assay. As a control, IL-2 was always incubated in the absence of cells in the same way (Abo et al. 1983).

EPICS™V analysis. Lymphocytes passing through nylon fiber columns were cultured in the presence of PHA for 12 hr, and were washed three times. Ten million cells were incubated with 50 μ l of monoclonal mouse anti-human IL-2R antibody (IgG₁ type) (Becton Dickinson monoclonal Center Inc., Mountain View, CA, USA) for 30 min at 4°C, and were washed three times. They were indirectly stained by the addition of 50 μ l of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG₁ (Biochemical Division, San Mateo, CA, USA) with a dilution of 1 : 50, and 20 μ l of PE-conjugated monoclonal anti-human T3 antibody, for 30 min, and were washed three times. For each sample, one to 2×10^6 cells were analyzed on a EPICS™V (Coulter Electronics, Inc., Hialeah, FL, USA) for IL-2R expression on the T cells. Most lymphocytes passing through nylon fiber columns were PE positive, suggesting that these cells were almost T lymphocytes. The negative control for IL-2R expression was calculated from the number of the unstained cells.

RESULTS

The response of MNC to IL-2. In the presence of both IL-2 and PHA, MNC from the healthy donors proliferated with the lapse of time, as monitored by ^3H -TdR incorporation. In order to remove the influence of PHA on IL-2 response, MNC were cultured with PHA for various time, washed three times and then, cultured with IL-2. Fig. 1 shows the time course of the IL-2 responsiveness of MNC previously treated with PHA. The cellular uptake of ^3H -TdR into MNC after 80 hr of culture in the presence of IL-2, increased from 3 hr of pretreatment with PHA, and reached the maximum after 12 hr, and it kept the similar value up to 48 hr. In the absence of IL-2, there was no increase in the uptake of ^3H -TdR even after the pretreatment time of as long as 48 hr.

Comparison of IL-2 responses of MNC from healthy donors and from cancer patients. The kinetics of the IL-2 response of MNC was compared between the healthy donors and the cancer patients. As indicated in Fig. 2, the proliferation

Fig. 1. The effects of IL-2 on the response to PHA of MNC. MNC from one healthy donor were cultured in the presence of 15 $\mu\text{g}/\text{ml}$ PHA at a concentration of 1×10^6 cells/ml for desired hr. After washing to remove PHA, 1×10^5 MNC were cultured again in microtiter wells with (●) or without (○) IL-2 (final 1 : 20) for additional 80 hr, and then, the radioactivity of ^3H -TdR was determined after 4 hr of labeling. Data points are means of triplicate experiments.

Fig. 2. The response to IL-2 of MNC from healthy donors or cancer bearers. MNC stimulated with PHA were cultured with IL-2 for various hr. The proliferative response was determined by the incorporation of $^3\text{H-TdR}$. Data points are means $\pm$ s.e. MNC were obtained from healthy donors (●) and from cancer patients (○).

of MNC from the cancer patients was lower than that from the healthy donors. In case of MNC from the healthy donors, the proliferation was started from 36 hr and the maximum response was observed at 96 hr after the incubation. In contrast, MNC from the cancer patients began to proliferate from 48 hr and was maximum at 108 hr. After that, the uptake of $^3\text{H-TdR}$ into MNC from both sources decreased with the lapse of time. The decrease in $^3\text{H-TdR}$ uptake might

Fig. 3. The effect of the medium change on IL-2 response of MNC. MNC were incubated with IL-2 for 4 days, and the medium was changed and IL-2 was added freshly. Then MNC were incubated additional 4 days. The proliferative response was estimated by incorporation of $^3\text{H-TdR}$. Broken lines indicate the uptake of $^3\text{H-TdR}$ into MNC without medium change. MNC obtained from a healthy donor (●) and from a cancer patient (○).

be due to the consumption of IL-2 in the culture medium.

As seen in Fig. 3, when the medium containing IL-2 was changed to the fresh one with IL-2 after 4 days of culture, the proliferation of MNC both from the healthy donors and from the cancer patients continued further. If the medium with IL-2 was changed more frequently, MNC continued to proliferate from about 3 weeks (data was not shown).

Analysis of the mechanism of the depression in IL-2 response of MNC from cancer patients. In order to investigate whether there is any difference in the IL-2 responses of MNC from the cancer patients and from the healthy donors, MNC were separated into lymphocytes and monocytes, and these separated cells were pretreated with PHA, and cultured with IL-2 for 96 hr. As shown in Fig. 4, the proliferation of MNC from the cancer patients was smaller than that from the healthy donors. In addition, the response of lymphocytes alone, unexpectedly, was reduced to 40 to 70% that of MNC by removing monocytes before culture with IL-2. This tendency was observed in lymphocytes from the cancer patients and also from the healthy donors. Monocytes alone reacted to IL-2 to lesser degree. After the pretreatment with PHA, MNC were fractionated into lymphocytes and monocytes, however, the IL-2 response of lymphocytes was reduced to the same level as described before.

To study the role of monocytes in the IL-2 response, monocytes from the

Fig. 4. The response of each fractionated MNC to IL-2. MNC were fractionated into lymphocytes and monocytes. After stimulation with PHA ($15 \mu\text{g/ml}$) for 12 hr, 1×10^5 each cells were cultured with IL-2 (1 : 20) for 96 hr, and the proliferation was measured by the incorporation of ^3H -TdR. Values are means of triplicated experiments. The values in the parenthesis represent the percent cpm of MNC ($\square$), lymphocytes (||||) and monocytes (|||||).

Fig. 5. IL-2 response of separated and reconstituted MNC subpopulations. MNC were fractionated into lymphocytes and monocytes, and MNC ($\square$), lymphocytes ($\square$) and monocytes ($\square$) were cultured with PHA ($15 \mu\text{g/ml}$) for 12 hr and then with IL-2 (1 : 20) under the same conditions as described in Fig. 4. The 3 columns on the left show the reactivities of the whole MNC and two subpopulations from a healthy donor (M.U.) and the three on the right show those from a cancer patient (K.I.). The cells of left bar in the middle ($\square$) consisted of monocytes from a cancer patient (K.I.) and lymphocytes from a healthy donor (M.U.) at the ratio of 1 : 2, and the right bar in the middle ($\square$) consisted of monocytes from a healthy donor (M.U.) and lymphocytes from a cancer patient (K.I.) at the ratio of 1 : 2. The result shows a representative one of three independent experiments, and the remaining results are quite similar to this. Values in parentheses are % of $^3\text{H-TdR}$ uptake in each cell fractions when the reactivity of MNC from a healthy donor is expressed as 100%. Value in brackets are % of $^3\text{H-TdR}$ uptake in each cell fractions when MNC from a cancer patient is expressed as 100%.

cancer patients were added to lymphocytes from the healthy donors with various proportion and then pretreated with PHA. Fig. 5 illustrates an example of the mutual response of these mixed cultures of lymphocytes and monocytes at a ratio of 2 to 1. As shown in this figure, the uptake of $^3\text{H-TdR}$ into lymphocytes from the healthy donors was not affected in the presence of monocytes from the cancer bearers. Furthermore, the IL-2 responsiveness of lymphocytes from the cancer bearers was not changed by addition of monocytes from the healthy donors. This tendency was always seen when the ratios of lymphocytes to monocytes in reconstituted cultures were changed from 2 : 1 to 5 : 1 (data not shown). From these results, we concluded that in cancer patients, the low response of lymphocytes might be responsible for the low response of MNC, and monocytes seemed to be required for sufficient proliferation of lymphocytes.

In order to know whether the extent of expression of IL-2R on lymphocytes of cancer patient is responsible for the lower response to IL-2, 5×10^6 to 2×10^7 lymphocytes treated with PHA were incubated with 0.2 ml of IL-2 for 24 hr at 37°C , and the supernatant was tested for the residual activity of IL-2. As shown in Fig. 6, the percent absorption of IL-2 to lymphocytes from the healthy donors increased as the number of lymphocytes increased, while in case from the cancer patients, no absorption was observed. As shown in Fig 7, IL-2R expression of each cell was determined from EPICS analysis of the cells by using the mono-

Fig. 6. Removal of IL-2 activity by peripheral lymphocytes. The aliquot of IL-2 (200 μ l, 1 : 10) was incubated with indicated numbers of lymphocytes from a healthy donor (●) or from a cancer bearer (○) for 24 hr at 37°C with occasional shaking. And the supernatant was used for the determination of absorption of IL-2 in the quantitative microwell assay. This figure shows a representative result of three independent experiments.

Fig. 7. The IL-2R expression of MNC from healthy donor and cancer patient. For EPICS analysis, lymphocytes from healthy donors and cancer patients passing through nylon fiber column were cultured with PHA (15 μ g/ml) and indirectly stained with monoclonal anti-human IL-2R antibody, followed by the addition of FITC-conjugated goat anti-mouse IgG and of PE-conjugated monoclonal anti human T3 antibody. The cells were obtained from: panel A, healthy donor; panel B, cancer patient. The proportions of T lymphocytes possessing IL-2R, were: 39.3% in a healthy donor, 17.4% in a cancer bearer. The representative result out of the three independent experiments is shown.

clonal anti-IL-2R antibody and the FITC-conjugated goat anti-mouse IgG. The number of T lymphocytes of healthy donor possessing IL-2R was much greater than that from the cancer patients, and a representative EPICS profile of three experiments is shown in Fig. 7. These results strongly suggested that the number of IL-2R on T lymphocytes of the cancer patients was similar to that of the healthy donors, but the number of T lymphocytes having IL-2R was greater in the healthy donors than in the cancer patients.

DISCUSSION

In this study, the response to IL-2 of MNC of the cancer patients and of the healthy donors were compared in vitro. Human peripheral MNC acquired the responsiveness to IL-2 and proliferated when previously treated with PHA. MNC began to proliferate after 3 hr and maximum proliferation was observed after 12 to 17 hr, and kept the same level of proliferation up to 48 hr after the addition of PHA. It might indicate that IL-2R on peripheral MNC emerged gradually 3 hr after stimulation with PHA, and was maximum after 12 to 48 hr. In this study, therefore, MNC were cultured for 48 hr, but it was not clear whether the expression of IL-2R was continued for such a long time of culture with PHA (Ly and Mischell 1974; Cantrell and Smith 1983). The responsiveness to IL-2 of MNC was decreased when the timing of the addition of IL-2 was too late after washing MNC to remove PHA (data was not shown).

IL-2 response of MNC was lower in cancer patients than that in healthy donors. This might be of problem when IL-2 is clinically used for the therapy of cancer. As an adoptive immunotherapy, the clinical applications of the passive transport of killer T cells specific to cancer antigens, which proliferated with IL-2 in vitro, are underway in this laboratory. However, the lower IL-2 response of lymphocytes from cancer patients may blockade therapeutic usefulness of the treatment with these effector cells. It was reported that the production of IL-2 of MNC from cancer patients has been lower than that from healthy donors, when cultured with IL-1, and that suppressor macrophages have been responsible for the lower response (Sasagawa et al. 1984). In order to examine the effect of monocytes on IL-2 response, MNC were separated into lymphocytes and monocytes. However, there was unexpectedly no suppressor activity in peripheral blood of cancer patients. Lymphocytes of cancer bearers themselves also could respond to a lesser degree, as compared with those from the healthy individuals.

Further, IL-2 response of lymphocytes fractionated from MNC decreased by 40 to 70% of whole MNC in both the cancer patients and the healthy donors. The expression of IL-2R on lymphocytes from cancer bearers was lower as shown by the absorption assay. Lymphocytes from healthy donors absorbed IL-2 in the medium for 24 hr at 37°C, however, lymphocytes from cancer bearers did not absorb it, suggesting that the total numbers of IL-2R on lymphocytes of cancer

patients were less than that of healthy donors. Moreover, EPICS analysis revealed that the number of T lymphocytes possessing IL-2R from the healthy donors was much greater than that from the cancer patients. Recently, it was reported that the activated human T cells have two distinct IL-2R with high and low affinities, as well as murine T cells have (Robb et al. 1984 ; Lowenthal et al. 1985). Smith and Cantrell (1985), and Depper et al. (1985) suggested that the cell growth signal of IL-2 can be transduced from high affinity receptors. In this study, the dilution of IL-2 was 50 U/ml to which both IL-2 receptors with high and low affinity, could bind. Furthermore, monocytes seemed to be required for sufficient proliferation of lymphocytes. The role of monocytes in IL-2 response is not clear yet. Kaye et al. (1984) reported that the essential cofactors for growth of one helper T cell line, which produced IL-2, were exogeneously given IL-1 and endogeneously produced IL-2 in mice. They postulated that the major role of IL-1 was to promote the expression of IL-2R on these T cells. In the present experimental system, monocytes play some essential role for promoting the expression of IL-2R on lymphocytes. The experiments are in progress about the role of monocytes in the response of lymphocytes to IL-2.

References

- 1) Abo, T., Miller, C.A., Balch, C.M. & Cooper, M.D. (1983) Interleukin 2 receptor expression by activated HNK-1 granular lymphocytes: A requirement for their proliferation. *J. Immunol.*, **131**, 1822-1826.
- 2) Böyum, A. (1968) Separation of lymphocytes from blood and bone marrow. *Scand. J. clin. Lab. Invest.*, **21**, Suppl. 97, 77-89.
- 3) Cantrell, D.A. & Smith, K.A. (1983) Transient expression of interleukin 2 receptors: Consequences for T cell growth. *J. exp. Med.*, **158**, 1895-1911.
- 4) Depper, J.M., Leonard, W.J., Drogula, C., Kronke, M., Waldmann, T.A. & Greene, W.C. (1985) Interleukin 2 (IL-2) augments transcription of the IL-2 receptor gene. *Proc. nat. Acad. Sci. USA*, **82**, 4230-4234.
- 5) Farrar, J.J., Benjamin, W.R., Hilfiker, M.L., Howard, M., Farrar, W.L. & Fuller-Farrar, J. (1982) The biochemistry, biology, and role of interleukin 2 in the induction of cytotoxic T cell and antibody-forming B cell responses. *Immunol. Rev.*, **63**, 129-166.
- 6) Gillis, S. & Smith, K.A. (1977) Long term culture of tumour-specific cytotoxic T cells. *Nature (Lond.)*, **268**, 154-156.
- 7) Gillis, S., Ferm, M.M., Ou, W. & Smith, K.A. (1978) T cell growth factor: Parameters of production and a quantitative microassay for activity. *J. Immunol.*, **120**, 2027-2032.
- 8) Kaye, J., Gillis, S., Mizel, S.B., Shevach, E.M., Malek, T.R., Dinarello, C.A., Lachman, L.B. & Janeway, C.A., Jr. (1984) Growth of a cloned helper T cell line induced by a monoclonal antibody specific for the antigen receptor: Interleukin 1 is required for the expression of receptors for interleukin 2. *J. Immunol.*, **133**, 1339-1345.
- 9) Larsson, E.-L. (1981) Mechanism of T cell activation. II. Antigen- and lectin-dependent acquisition of responsiveness to TCGF is a nonmitogenic, active response of resting T cells. *J. Immunol.*, **126**, 1323-1326.
- 10) Larsson, E.-L. & Coutinho, A. (1979) The role of mitogenic lectins in T-cell triggering. *Nature (Lond.)*, **280**, 239-241.

- 11) Lowenthal, J.W., Zubler, R.H., Nabholz, M. & MacDonald, H.R. (1985) Similarities between interleukin-2 receptor number and affinity on activated B and T lymphocytes. *Nature (Lond.)*, **315**, 669-672.
 - 12) Ly, I.A. & Mischell, R.I. (1974) Separation of mouse spleen cells by passage through columns of Sephadex G-10. *J. Immunol. Meth.*, **5**, 239-247.
 - 13) Morgan, D.A., Ruscetti, F.W. & Gallo, R. (1976) Selective in vitro growth of T lymphocytes from normal human bone marrows. *Science*, **193**, 1007-1008.
 - 14) Mosier, D.E. (1967) A requirement for two cell types for antibody formation in vitro. *Science*, **158**, 1573-1575.
 - 15) Robb, R.J., Greene, W.C. & Rusk, C.M. (1984) Low and high affinity cellular receptors for interleukin 2: Implications for the level of Tac antigen. *J. exp. Med.*, **160**, 1126-1146.
 - 16) Sasagawa, Y., Kohgo, Y., Sakamaki, S., Onodera, Y., Niitsu, Y. & Urushizaki, I. (1984) Mechanism of immunosuppressive action of peripheral monocytes of cancer patients. *Shinryo to Shinyaku*, **21**, 439-444. (Japanese)
 - 17) Schroff, R.W., Gottlieb, M.S., Prince, H.E., Chai, L.L. & Fahey, J.L. (1983) Immunological studies of homosexual men with immunodeficiency and Kaposi's sarcoma. *Clin. Immunol. Immunopath.*, **27**, 300-314.
 - 18) Smith, A.K. (1980) T-cell growth factor. *Immunol. Rev.*, **51**, 337-357.
 - 19) Smith, K.A. & Cantrell, D.A. (1985) Interleukin 2 regulates its own receptors. *Proc. nat. Acad. Sci. USA*, **82**, 864-868.
 - 20) Tan, T. & Gordon, J. (1971) Participation of three cell types in the anti-sheep red blood cell response in vitro. *J. exp. Med.*, **133**, 520-533.
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