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COMPARISON OF THE BIOLOGICAL PROPERTIES OF PURIFIED NATURAL AND RECOMBINANT HUMAN INTERLEUKIN-2

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Summary : We compared the biological properties of the purified recombinant human IL-2 derived from E. coli with those of purified natural IL-2. Both had almost the same specific in vitro activities on a weight basis to (i) support long-term proliferation of IL-2 dependent human peripheral blood lymphocytes, a mouse killer T cell line, and a mouse natural killer cell line; (ii) induce killer cells in normal mouse spleen cells; and (iii) induce antibody forming cells in nude mouse spleen cells. No differences in these biological activities were found between two forms of natural IL-2 that were separable by reverse phase high performance liquid chromatography.

Interleukin-2 (IL-2), first described as TCGF by Morgan et al. (1), is produced by T lymphocytes after stimulation with mitogens or antigens (2,3) and has the ability to promote and maintain in vitro long-term cultures of T cells (1,4). Recent studies from several laboratories indicate that this factor also promotes in vitro long-term proliferation of natural killer cell lines (5), enhances thymocyte mitogenesis (6), induces cytotoxic T cells and plaque forming cells (PFC) in cultures of nude mouse spleen cells (7,8), and induces interferon (9). These facts suggest that IL-2 may be useful for augmenting immune responses and restoring deficiencies in cellular and humoral immunity to normal levels. Furthermore, IL-2 production and response are important parameters of immunological functions and their measurement may be useful in clinical diagnosis of aberrant immune status (10,11).

Abbreviations used in this paper : IL-2, interleukin-2 ; TCGF, T cell growth factor ; PFC, plaque forming cell ; kDa, kilodalton ; Con A, concanavalin A ; TPA, 12-O-tetradecanoylphorbol-13-acetate ; nIL-2, natural interleukin-2 ; rIL-2, recombinant interleukin-2 ; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis ; RP-HPLC, reverse phase high performance liquid chromatography ; PBL, peripheral blood lymphocytes ; NK cell, natural killer cell ; FCS, fetal calf serum ; ^{3}H-TdR, ^{3}H-thymidine ; SRBC, sheep red blood cells ; MLC, mixed lymphocyte culture.

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Natural human IL-2 is a group of glycoproteins with molecular masses of 13-17.5 kDa (12-17), and consists of multiple isoelectric forms of pI ranging from 6.6 to 8.2 (17,18). The heterogeneity in molecular weight of natural IL-2 may depend on a variable degree of glycosylation (18).

Studies of the actual biological role of this lymphokine have been hampered by the limited amount of purified natural IL-2 obtainable from activated lymphocytes (2), T cell hybridomas (19), or even from high producer cell lines such as Jurkat (20). Recently, human IL-2 cDNAs, derived from a T cell leukemia (Jurkat) and spleen cells activated by phytohemagglutinin and TPA, were cloned and expressed in eukaryotic cells and E. coli (21-23). We also have succeeded in cloning IL-2 cDNA using mRNA (24) derived from human PBL stimulated by ConA and TPA, having the IL-2 structural gene expressed in E. coli, and purifying recombinant DNA-derived IL-2 (rIL-2). Natural human IL-2 (nIL-2) derived from PBL stimulated by ConA and TPA was recently purified to apparent homogeneity as judged by SDS-PAGE and RP-HPLC (17).

In the studies reported here, we compared the in vitro biological properties of the purified rIL-2 (nonglycosylated) with those of two purified molecular species of nIL-2 (glycosylated) which were separated by RP-HPLC and demonstrated that all three have similar biological properties.

MATERIALS AND METHODS

Animals : Female DBA/2, C57BL/6, and BALB/c nu/nu mice, aged 6-8 weeks, were used. They were bred and maintained at the Drug Safety Research Laboratories, Central Research Division, Takeda Chemical Industries, Ltd.

Human PBL conditioned medium : Human PBL conditioned medium was prepared as described previously (24,25). Briefly, human PBL were separated from buffy coat cells by dextran. A suspension of leukocytes ($5x10^6$ cells/ml) in RPMI-1640 medium containing 10% heat-inactivated FCS (M. A. Bioproducts, Maryland) was induced to produce IL-2 by combined treatments with TPA (15 ng/ml) and ConA (40 µg/ml) (P-L Biochemicals, Inc., Milwaukee, USA). After the cell suspension was incubated at 37° C for 72 hr, the culture supernatants were collected by centrifugation. This solution is designated as "crude nIL-2".

Cell lines : IL-2 dependent mouse killer cell line (NRB) (26) was kindly provided by Dr. Eiichi Nakayama of the Center for Adult Diseases, Osaka. NRB was maintained in the presence of rat spleen conditioned medium (27). The IL-2 dependent mouse NK cell line (NKC3) (5), maintained in the presence of human PBL conditioned medium, was kindly provided by Dr. Katsuo Kumagai of Tohoku University. A human IL-2 dependent PBL line was established as described by Morgan et al. (1). Briefly, human PBL ($2x10^6$ cells), collected by LSM™ (Litton Bionetics, Inc., Kensington), were cultured in a Corning *tissue culture flask* #25100 in RPMI-1640 medium containing 20% heat-inactivated FCS and 30% human PBL conditioned medium. After one week, the density of viable cells was adjusted to $2x10^5$ cells/ml with the same medium and cultured. The viable cells were passaged every 4 or 5 days.

Assay for IL-2 activity : IL-2 activity was determined by the microassay method using the IL-2 dependent murine NKC3 line as described previously (24).
Purified nIL-2 and rIL-2 : The nIL-2 derived from human PBL stimulated by ConA and TPA was purified by sequential chromatography by using SP-Sephadex C-25, DEAE-Sephacel, Ultrogel AcA-54 gel filtration and RP-HPLC according to the method of Kato et al. (17). The DE-52 step was omitted. On RP-HPLC nIL-2 was resolved into two peaks of activity; the earlier peak was designated as P-1, the later peak was designated as P-2 in this paper. P-1 was found to consist of three molecular species of nIL-2, A-1, B-1 and C-1 described by Kato et al. (17), and P-2 was found to consist of A-2, B-2 and C-2. P-1 and P-2 contained no contaminating proteins as judged by SDS-PAGE. rIL-2 was extracted from E. coli having the gene coding for human IL-2 and purified to apparent homogeneity by cation exchange chromatography on CM-Toyopearl 650M (Toyo Soda Manufacturing Co., Ltd. Tokyo) and RP-HPLC. The purified preparation migrated as a 15 kDa protein on SDS-PAGE and its purity was estimated to be more than 99.8 % (Fig.1).
SDS-PAGE : SDS-PAGE was carried out with a 15% polyacrylamide gel under reducing conditions according to the method of Laemmli (28).
Incorporation of ^{3}H-TdR : IL-2 dependent cell lines (human PBL, NRB, and NKC3) were washed and resuspended in RPMI-1640 medium containing 20% heat-inactivated FCS at $6x10^{5}$ cells/ml. Test samples containing IL-2 (50 µl) were serially diluted two-fold on 96 well flat-bottomed microplates (A/S Nunc, Roskilde, Denmark), and the suspension (50 µl) of each of the IL-2 dependent cell lines was added to each well. One µCi of ^{3}H-TdR (25 Ci/mmol) in 2 µl of the medium was added to each well at 20 hr (in case of human PBL cell line; 68 hr) after the addition of IL-2, and 4 hr later the cells were harvested using an automatic cell harvester. Incorporation of ^{3}H-TdR was counted in a scintillation spectrometer.
In vitro killer cell induction : BALB/c mouse spleen cells ($2.5x10^{6}$ cells), collected by LSM™, were cultured in 24 well flat-bottomed dishes (Linbro #76-033-05, Virginia) in 2 ml of RPMI-1640 medium supplemented with 10% heat-inactivated FCS, 4 mM glutamine, 1 mM sodium pyruvate, 100 µg/ml kanamycin, 2 mg/ml of sodium bicarbonate, $5x10^{-5}$ M 2-mercaptoethanol, and 25 mM 2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (complete RPMI medium), with or without $7.5x10^{5}$ gamma-irradiated (2000 rad.) C57BL/6 mouse spleen cells collected by LSM™. After being incubated 4 days at 37° C in a humidified atmosphere of 5% CO_2 in air, the cells were washed and assayed for cytotoxic activity against EL-4 and YAC-1 target cells in a 4 hr ^{51}Cr-release assay (29). EL-4, a leukemic cell line derived from C57BL/6, is resistant to NK cells. YAC-1 is a leukemic cell line sensitive to NK cells.

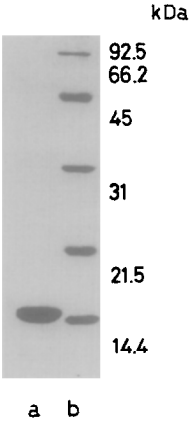


Fig. 1. SDS-PAGE of the purified rIL-2. SDS-PAGE was carried out with a 15% polyacrylamide gel under reducing conditions. Proteins were stained with Coomassie Brilliant Blue R250. Lane a, rIL-2 (10 µg); lane b, marker proteins (2 µg each).

In vitro antibody response : BALB/c nu/nu mouse spleen cells ($1x10^6$ cells), collected by LSM™, were cultured in Falcon plastic tubes # 2054 in 0.2 ml complete RPMI medium with SRBC ($0.5x10^6$ cells) at 37° C in a humidified atmosphere of 5% CO_2 in air. Various doses of IL-2 were added at the beginning of the cultures. On day 5, the cells were harvested, and the number of direct PFC was determined by the technique of Cunningham and Szenberg (30). Results were presented as the mean PFC of triplicate cultures.

RESULTS

1. Growth enhancement of IL-2 dependent cell lines

1-(1). Enhancement of the ^{3}H-TdR incorporation

We compared the ability of the purified rIL-2, crude nIL-2, and purified nIL-2 (P-1 and P-2 ; see MATERIALS AND METHODS), to enhance ^{3}H-TdR incorporation into three IL-2 dependent cell lines: the human IL-2 dependent PBL, a mouse killer T cell line (NRB), and a mouse NK cell line (NKC3). As shown in Fig.2, all the IL-2 preparations enhanced ^{3}H-TdR incorporation in the three IL-2 dependent cell lines in a quite similar way.

1-(2). Long-term culture

The same IL-2 preparations were examined for their ability to maintain the long-term culture of the three IL-2 dependent cell lines. As shown in Fig.3, each of these IL-2 preparations maintained the cell lines in culture for at least 20-45 days. Cells could not be maintained without rIL-2 or nIL-2.

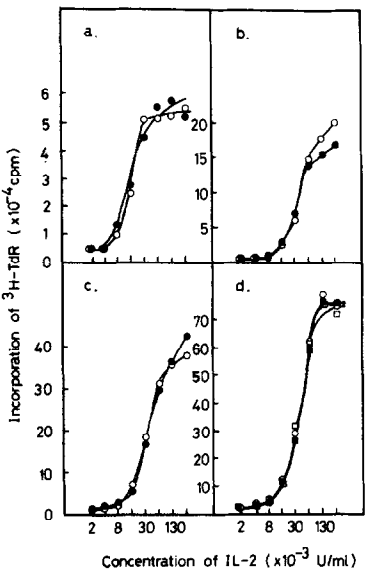
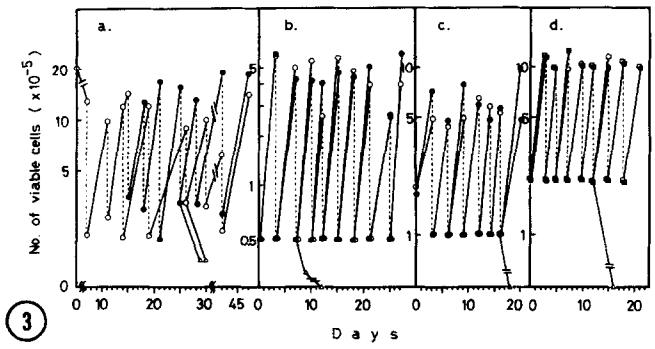


Fig. 2. Effect of crude nIL-2, purified nIL-2 (P-1, P-2), and purified rIL-2 on ^{3}H-TdR incorporation in three IL-2 dependent cell lines: a, human PBL; b, mouse killer T cell line (NRB); c and d, mouse NK cell line (NKC3).
(●: purified rIL-2, ○: crude nIL-2, ■: purified nIL-2(P-1), □ :purified nIL-2(P-2))



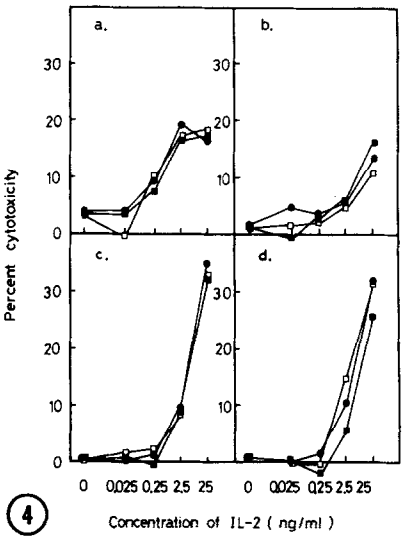


Fig. 3. Effect of crude nIL-2, purified nIL-2, and purified rIL-2 on the maintenance of three IL-2 dependent cell lines in culture: a, human PBL; b, mouse killer T cell line (NRB); and c and d, mouse NK cell line (NKC3). Cells were cultured in the presence of IL-2 .
(● : purified rIL-2, ○ : crude nIL-2, ■ : purified nIL-2(P-1), □ :purified nIL-2 (P-2), or in the absence of IL-2 (△) as shown in MATERIALS AND METHODS. In cases of a, c and d, 1.1 U/ml and b, 0.33 U/ml of IL-2 were used.)

Fig. 4. Effect of the nIL-2 and rIL-2 on the generation of the killer cells. BALB/c mouse spleen cells were cultured with (a,c) or without (b,d) irradiated C57BL/6 mouse spleen cells. After 4 days, killer cell activities were determined as shown in MATERIALS AND METHODS by cytotoxicity against EL-4 (a and b) and YAC-1 (c and d) at effector/target ratio of 20/1 in ^{51}Cr-release assay.
(● : purified rIL-2, ■ : purified nIL-2 (P-1), □ : purified nIL-2 (P-2))

2. Induction of killer cells in vitro

The effects of the purified nIL-2 and rIL-2 on the generation of MLC specific killer cells and the NK cells, using EL-4 and YAC-1 cells as targets, respectively, were examined. As shown in Fig.4, both purified rIL-2 and nIL-2 (P-1 and P-2) induced nonspecific and MLC specific killer cells depending on the dose of purified rIL-2 and nIL-2. No significant difference in specific activities on a weight basis was observed. The optimal dose of IL-2 in the induction of the MLC specific killer cells was around 2.5 ng/ml.

3. Enhancement of in vitro primary antibody reponse.

T cell-deficient BALB/c nu/nu mice do not repond to SRBC, T cell dependent antigen, to produce antibody. However, as shown in Fig.5, when purified rIL-2 or nIL-2 (P-1 and P-2) was added to the nude mouse spleen cell cultures with SRBC, an increase in the number of the PFCs was observed depending on the dose of the purified rIL-2 or nIL-2.

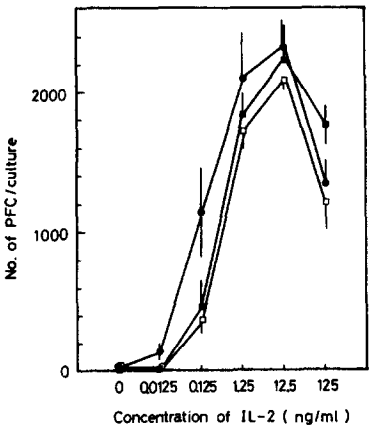


Fig. 5. Effect of purified nIL-2 and rIL-2 on in vitro primary anti-SRBC antibody response. BALB/c nu/nu mouse spleen cells were mixed with SRBC and cultured with various concentrations of purified nIL-2 or rIL-2. PFCs were determined as described in MATERIALS AND METHODS. Points represent the mean ± S.E. of triplicate cultures. (● : purified rIL-2, ■ : purified nIL-2(P-1), □ : purified nIL-2(P-2))

The optimal dose of IL-2 in enhancing the PFCs was between 1.25 and 12.5 ng/ml. In this case also, the specific activities were almost the same.

DISCUSSION

In this paper, we have shown that, on a weight basis, purified rIL-2 has almost the same in vitro biological specific activities as purified nIL-2. The rIL-2 is nonglycosylated, whereas the nIL-2 from Jurkat is reported to be glycosylated at the position 3 (threonine) of the primary amino acid sequence (16); nIL-2 molecules from the tonsils and PBL are supposed to contain more carbohydrates than that from Jurkat (16,18). This suggests that the carbohydrate moieties of the nIL-2 could not be responsible for its in vitro biological activities; a recent paper by Rosenberg et al. confirmed this point (23), although they did not compare directly the biological activities of rIL-2 with those of nIL-2. It is clear from our results that TCGF activity, the induction of killer cell activity, and the induction of PFC responses in cultures of nude mouse spleen cells are attributable to a single nonglycosylated molecule. We also showed that two molecular species of purified nIL-2 (P-1 and P-2) possess the same specific activities. It is an interesting observation that purified IL-2 supported the long-term growth of human PBL without stimulation, except for the first, of mitogens during culture, because it is reported that the expression of the IL-2 receptor on the cell

surface by mitogens is a transient but necessary event for cells to respond to IL-2 (31). Killer cells induced by purified IL-2 seem to include specific killer cells and nonspecific killer cells; the latter include NK cells and other nonspecific killer cells generally termed lymphokine activated killer cells because Meth A cells and P815 cells are also killed under the system described in Fig. 4. (data not shown). Other biological activities on human PBL of the purified rIL-2 not shown in this paper include stimulation of antibody production, induction of IFN-γ, and enhancement of natural killer cell activities induced by IFN-γ.

With the advent of recombinant DNA technology and success in bulk fermentation of E. coli harboring the human IL-2 gene, it has become possible to produce large amounts of IL-2 and to study the precise molecular characterization of this lymphocyte regulatory molecule both in vitro and in vivo.

We hope that this rIL-2 is useful in augmenting the low level immune response in immune deficient patients to normal levels of cellular and humoral immunity and is applicable to patients with infectious diseases, acquired immunodeficiency syndrome, autoimmune diseases, or cancer (32-34).

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