

Antitumor activity of interleukin-21 prepared by novel refolding procedure from inclusion bodies expressed in *Escherichia coli*

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Received 26 June 2002; revised 2 August 2002; accepted 12 August 2002

First published online 23 August 2002

Edited by Judit Ovádi

Abstract Interleukin-21 (IL-21) has recently been identified as a novel 4-helix-bundle type I cytokine possessing a cytokine receptor γ chain essential for the immune response. We report the preparation and functional characterization of *Escherichia coli*-expressed recombinant human IL-21 (rIL-21). The rIL-21, expressed as insoluble inclusion bodies in *E. coli*, was solubilized and then refolded by using a modified dialysis method. The introduction of redox reagents during refolding led to a dramatic increase in the refolding efficiency. Circular dichroism spectrum analysis showed that the refolded rIL-21 had an α -helix as a secondary structure, which is a characteristic of type I cytokines. Flow cytometry confirmed previous reports that rIL-21 binds to CD3-activated T cells (T-LAK) and to cell lines Raji, HL60, and Jurkat. rIL-21 stimulated the proliferation of T-LAK but not peripheral blood mononuclear cells, and this effect seems to be identical to that of co-stimulation with anti-CD3 antibody. Growth inhibition assay indicated that enhancement of the cytotoxicity of T-LAK to the human bile duct carcinoma TFK-1 depended on the concentration of rIL-21. Thus, refolded rIL-21 had activity identical to that of authentic IL-21 and enhanced the anti-tumor activity of T-LAK. These conclusions suggest the potential use of the refolded cytokine in adoptive immunotherapy using T-LAK cells and in the discovery of other functions of the cytokine. © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Interleukin-21; Refolding; Inclusion body; Adoptive immunotherapy; LAK

1. Introduction

Interleukin-21 (IL-21) was identified in 2000 [1]. It belongs

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Abbreviations: IL-21, interleukin-21; rIL-21, recombinant interleukin 21; Fv, antibody variable domain fragment; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; T-LAK, lymphokine-activated killer with T cell phenotype; PBMC, peripheral blood mononuclear cell; PCR, polymerase chain reaction; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt; E:T, effector:target ratio; BrdU, bromodeoxyuridine; FITC, fluorescein isothiocyanate; ELISA, enzyme-linked immunosorbent assay; Ab, antibody; mAb, monoclonal antibody

to the type I cytokine family and has homology to IL-2, IL-4, and IL-15. Its receptor, IL-21R, has marked sequence homology to the γ chain of IL-2R. IL-21 has a role in the proliferation and maturation of natural killer cells from bone marrow, in the proliferation of mature B cells under co-stimulation with anti-CD40 monoclonal antibody (mAb), and in the proliferation of T cells under co-stimulation with anti-CD3 mAb [1]. It shares a common cytokine receptor γ chain, which is essential for the immune response, with IL-2, IL-4, IL-7, IL-9, and IL-15 [2]. Although the γ chain is dispensable for the binding of IL-21 to IL-21R, it is essential for the signaling by IL-21R of the activation of Janus kinase (JAK) 1 and JAK3, followed by phosphorylation of STAT1 and STAT3 [2]. These facts indicate that the γ chain is an indispensable subunit of IL-21R, as seen in IL-2 signaling via IL-2R binding. Because IL-21 signaling has not been functionally characterized, a highly efficient system for preparing IL-21 would be useful to researchers in immunology and medicine. In addition, the cytokine may be useful for adoptive immunotherapy through its use in activating T cells.

Recent advances in genetic engineering have made it easier to prepare recombinant proteins such as cytokines, chemokines, co-stimulatory molecules, and fragments of variable regions of antibodies (Fv) [3–6]. The successful construction of several proteins by using bacterial expression systems has been reported, and some proteins are now used for therapeutic purposes [7–9]. Overproduction of proteins, however, often leads to the formation of insoluble aggregates, referred to as inclusion bodies, in the cytoplasmic or periplasmic space. In many cases, the inclusion bodies are made of the expressed proteins. If these proteins can be refolded, large quantities can be made available for industrial and therapeutic needs [10,11]. Therefore, a stable and convenient refolding system would benefit the preparation of recombinant proteins.

Here, we describe the successful construction of recombinant human IL-21 (rIL-21) from *Escherichia coli* inclusion bodies comprising highly expressed proteins that were refolded into the functional form by stepwise dialysis. rIL-21 may prove useful in adoptive immunotherapy using T-LAK cells and in the investigation of novel functions and effects of IL-21.

2. Materials and methods

2.1. Construction of rIL-21 expression vector

A completely synthesized gene for rIL-21 was made from six oligonucleotides by using an overlap-extension polymerase chain reaction

(PCR) method as described previously [12,13]. In brief, the oligonucleotides were approximately 90 bp in length and had overlapping regions of about 20 bp (Table 1). These oligonucleotides were designed using codons that exist with high frequency in the *E. coli* genome (Table 1). The six oligonucleotides were assembled, and the full-length DNA was amplified by PCR (Fig. 1). A 2nd PCR used two external primers and the DNA fragments amplified by the 1st PCR as templates. The final PCR product was digested with *Nco*I and *Sac*II and cloned into a T7 promoter-based pUT vector [10].

2.2. Expression and purification of rIL-21

rIL-21 was expressed and purified as described previously [14]. In brief, *E. coli* strain BL21 (DE3) transformed with rIL-21 expression vector was incubated at 37°C in LB broth. When the optical density reached 0.8 at 600 nm, 1 mM isopropyl-1-thio- β -D-galactopyranoside was added to the culture to induce protein production, and the cells were grown overnight. Cell pellets separated from the culture by centrifugation (2000g, 35 min) were resuspended in 10 ml phosphate-buffered saline (PBS), ultrasonicated at 150 W for 15 min, and centrifuged at 4500g for 20 min. Then the separated intracellular insoluble fraction was solubilized and denatured with 10 ml of 6 M guanidine-HCl in PBS overnight at 4°C. After solubilization, the proteins were purified in a Talon metal affinity resin column (Clontech, Palo Alto, CA, USA).

2.3. Refolding of rIL-21 by stepwise dialysis

We have previously shown the preparation system of functional soluble antibody fragments from this insoluble aggregate using the stepwise dialysis in vitro refolding system [15]. Thus, here we used a modified method to obtain functional rIL-21 from the intracellular insoluble fraction. Purified rIL-21 was diluted to 7.5 μ M with 6 M guanidine-HCl in 50 mM Tris-HCl (pH 8.0), and stepwise dialysis was used to remove the denaturant as previously reported [15]. To obtain soluble rIL-21, we optimized the refolding system of a previous report [10]. The refolding procedure is summarized in Fig. 2. To increase the refolding efficiency, we added 3.75 mM reducing reagent (reduced form of glutathione; Sigma, St. Louis, MO, USA) and 375 μ M oxidizing reagent (oxidized form of glutathione; Sigma) to the refolding solution when the concentration of guanidine-HCl was 1 and 0.5 M for shuffling the disulfide bonds.

2.4. Gel filtration

Gel filtration of refolded rIL-21 was performed using a Superdex 75 column (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) (2.6 \times 60 cm) connected to an FPLC system pre-equilibrated with PBS. 10 ml of refolded protein was subjected to gel filtration at a flow rate of 3.0 ml/min.

2.5. SDS-PAGE and Western blotting

The analysis used 500- μ l aliquots of culture supernatant. The total protein in each fraction, precipitated with 6% trichloroacetic acid and 0.083% deoxycholate, was submitted to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions as previously reported [16] and stained with Coomassie brilliant blue R-250. Then the proteins in the gel were blotted onto nitrocellulose membranes (Amersham) and incubated with peroxidase-conjugated anti-His tagged mAb (Invitrogen). The ECL Detection System (Amersham) was used for signal enhancement.

2.6. Circular dichroism (CD) spectrum measurement

CD spectra were measured with an AVIV Circular Dichroism Spectrometer with a 0.1-cm quartz cuvette for the near-UV spectrum and a 1-cm quartz cuvette for the far-UV spectrum. The conditions were 1-nm band width, 4-s averaging time, and four scans.

2.7. Preparation and stimulation of effector cells

For induction of T-LAK cells, peripheral blood mononuclear cells (PBMCs) donated by a healthy volunteer and isolated by density-gradient centrifugation were cultured for 48 h in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μ g/ml streptomycin and 100 IU/ml recombinant human IL-2 (kindly supplied by Shionogi Pharmaceutical, Osaka, Japan) at a cell density of 1×10^6 /ml in a culture flask (A/S Nunc, Roskilde, Denmark) pre-coated with OKT3 mAb (mouse IgG2a) (10 μ g/ml). The induced T-LAK cells were then transferred to another flask and expanded in culture medium containing 100 IU/ml recombinant human IL-2 for 2–3 weeks [17].

2.8. Flow cytometric analysis

Test cells (5×10^5) were incubated for 30 min on ice with 100 μ l rIL21 (about 100 μ g/ml). After washing in PBS plus 0.1% NaN₃, they were exposed to 9E10 anti-c-myc mAb (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and then fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG, both for 30 min on ice. The stained cells were analyzed by flow cytometry (FACS Calibur; Becton Dickinson, San José, CA, USA).

2.9. Proliferation assay of PBMCs and T-LAK cells

Proliferation of mitogen-activated human PBMCs and T-LAK cells was assessed by using a Cell Proliferation enzyme-linked immunoassay (ELISA) system (Amersham). Briefly, 1×10^5 freshly isolated PBMCs suspended in 0.2 ml culture medium were added to each well of 96-well flat-bottomed plates (Sumitomo Bakelite, Osaka, Japan) in the presence of various concentrations (from 0.1 to 100 ng/ml) of rIL-21. After incubation for 48 h at 37°C, 5-bromo-2'-deoxyuridine (BrdU) labeling reagent was added, and the cells were incubated for an additional 24 h. Cell labeling was detected according to the manufacturer's assay protocol for cells in suspension. The optical density was measured by using a plate reader (model 3550; Bio-Rad, Tokyo, Japan) at 450 nm.

2.10. In vitro growth inhibition assay

The enhanced anti-tumor effects of the constructed rIL-21 were evaluated in a growth inhibition assay using various cell lines (i.e. human bile duct carcinoma TFK-1, human lung carcinoma OBA-LK1 and human colon cancer DLD-1) as a target and T-LAK cells or PBMCs as an effector. In vitro growth inhibition assays were performed with a 3-(4,5-dimethylthiazole-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) assay kit (CellTiter 96; aqueous, non-radioactive cell proliferation assay; Promega, Madison, WI, USA). Target cells (5000 cells in 100 μ l culture medium) were added to 48 wells of a 96-well flat-bottomed plate (Costar, Cambridge, MA, USA). The cells were cultured overnight to allow adhesion to the bottom of the wells. After removal of the culture medium by aspiration, 100 μ l of T-LAK cells or PBMCs plus various concentrations (from 1.25 to 10 ng/ml) of rIL-21, or 100 μ l of rIL-21 alone, was added to each well. After culture for 48 h at 37°C,

Table 1
Primers of overlap-extension PCR for whole synthesized IL-21 gene

1st PCR primers	
(1)	5'-NNNCCATGGCCAGGATCGCCATATGATTTCGCATGCGTCAGCTGATTGATATTGTTGATCAGCTGAAAAACTATGTGAACGACTTGGTGC-3'
(2)	5'-GAGCGCCTTTAGCTGCTT+CAGAAAGCGCAGCTGAAAATCTGCGAATACCGGCAACAATCAACGCATCATTAATGTGAGCATT-3'
(3)	5'-CAGAAACATCGTCTGACCTGCCCGTCATGTGATCTTTATGAAAAAAGCCGCCGAAAGAAATTCTGGAACGTTTCAAATCTCTT-3'
(4)	5'-AAAGCAGCTAAAGCGCTCCATTCACAGTTGGTTTCCACATCTTCCGGGGCCGCAGAAATCCGGCACCAAGTCGTTCCACATA-3'
(5)	5'-AGGTGACAGCATGTTTCTGGCGACGACCGGCATTGGTAGACGGCGGTTTACGTTTCAGCTTTTAAATGCTCACATTAATGATGCG-3'
(6)	5'-NNNCCGGCGCCGAGAATCTTCACTGCCGTGGTGCGAGAGCTCAGATGCTGGTGGATCATTTTTTGCAGAAAGAGATTGAAACGTTCCAGA-3'
2nd PCR primers	
reverse primer	5'-NNNCCATGGCCAGGATCGCCATA-3'
forward primer	5'-NNNCCGGCGCCGAGAATCTTCA-3'

Numbering of 1st PCR primers correspond to Fig. 1. The italic letters of each primers show restriction enzyme sites and underlined letters indicate overlap regions.

each well was washed three times with PBS to remove effector cells and dead target cells. This step was followed by the addition of 95 μ l/well of culture medium and 5 μ l/well of a fresh mixture of MTS/phenazine methosulfate solution (Promega). The plates were incubated for 1 h at 37°C and then read on a microplate reader (Bio-Rad model 3550) at 490 nm. Growth inhibition of target cells was calculated as follows: % growth inhibition = $[1 - (A_{490} \text{ of experiment} - A_{490} \text{ of background}) / (A_{490} \text{ of control} - A_{490} \text{ of background})] \times 100$ [18].

3. Results

3.1. Expression of recombinant IL-21 as inclusion body in *E. coli*

Recombinant IL-21 (rIL-21) was expressed in *E. coli* strain BL21 (DE3). SDS-PAGE and Western blotting using anti-His-tagged Ab showed that rIL-21 existed mainly in the intracellular insoluble fraction (Fig. 3a, b).

3.2. Purification and refolding of rIL-21

SDS-PAGE analysis revealed highly purified rIL-21 as a single band with an estimated size of 18.4 kDa (Fig. 3c). The refolding efficiency was about 57% with oxidizing reagent alone but 90% with oxidizing and reducing reagents. This result suggests the critical role of the shuffling of disulfide bonds during refolding. Gel filtration analysis indicated the monomeric structure of the refolded protein (Fig. 4). The refolded rIL-21 did not aggregate even after incubation at 37°C for a week (data not shown). Thus, the folding was stable. The final yield of refolded IL-21 was >200 mg/l of culture.

3.3. CD spectrum measurement

The troughs at 208 and 222 nm in the far-UV spectrum (Fig. 5a) show that rIL-21 has an α -helix-rich secondary structure. This result corresponds well with the previous prediction that IL-21 has a 4-helix-bundle structure because of its primary amino acid sequence and the characteristic structure of a type I cytokine [1]. The near-UV spectrum (Fig. 5b) suggested the interaction of an aromatic residue in the tertiary structure of the protein. These results show that the rIL-21

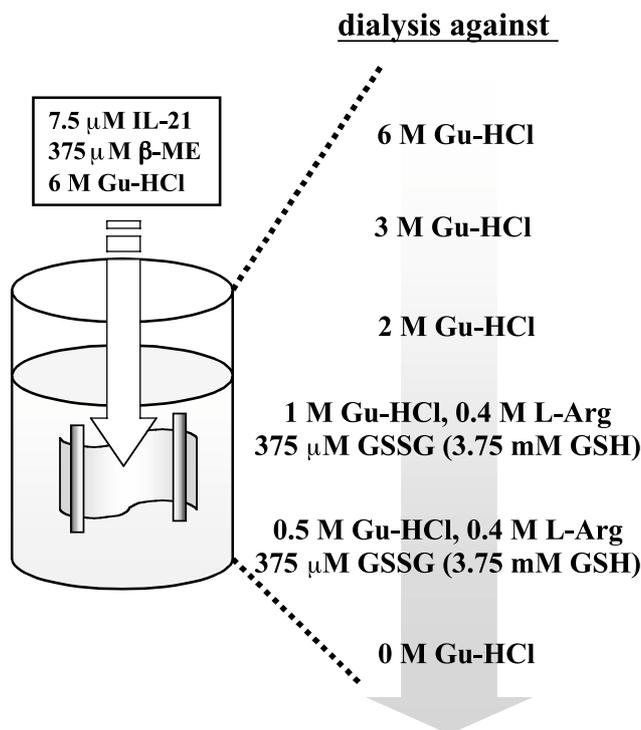


Fig. 2. Procedure for refolding *E. coli*-expressed recombinant IL-21. See details in Materials and Methods. The buffer solution during refolding is 50 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA (pH 8.0, 4°C). Each dialysis step lasts 6–12 h. The concentration of reducing reagent added for disulfide shuffling is shown in parentheses. GSSH, oxidized form of glutathione; GSH, reduced form of glutathione; L-Arg; L-arginine.

prepared by disulfide shuffling was refolded and contains an α -helix as a secondary structure.

3.4. Flow cytometric analysis

The binding activity of rIL-21 to IL-21R was confirmed by flow cytometry using IL-21R-positive cell lines and lymphokine-activated T cells (T-LAK cells). Refolded rIL-21 bound

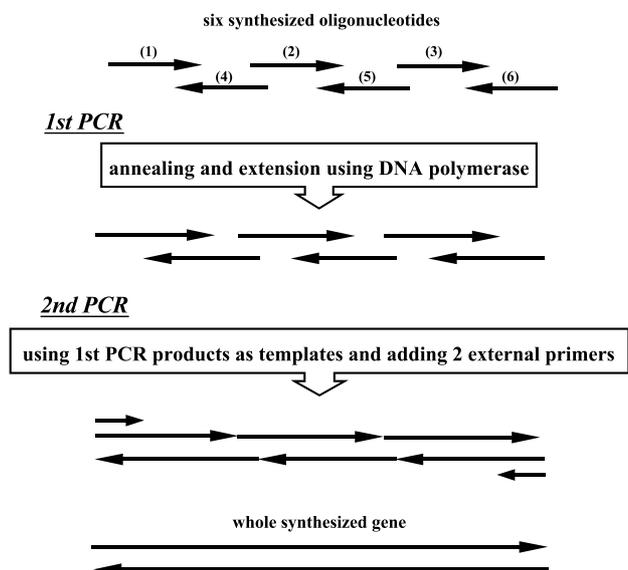


Fig. 1. Construction of genes encoding IL-21 by overlap-extension PCR. See details in text.

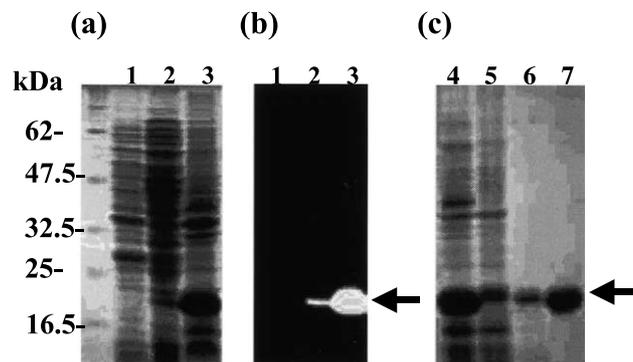


Fig. 3. Expression and purification of recombinant IL-21. (a) SDS-PAGE under reducing conditions and (b) Western blot analysis by anti-His-tagged mAb of fractions of *E. coli* BL21 (DE3) cells expressing rIL-21. Lane 1, proteins in the bacterial supernatant fraction; 2, proteins in the intracellular soluble fraction; 3, proteins in the intracellular insoluble fraction (inclusion bodies). (c) SDS-PAGE under reducing conditions of fractions separated by immobilized metal affinity chromatography under denaturing conditions. Lane 4, total proteins in the insoluble fraction; 5, flow-through fraction; 6, wash fraction; 7, eluted fraction.

to IL-21R-positive cells, Raji cells (human Burkitt lymphoma), HL-60 cells (human blood promyelocytic leukemia), and Jurkat cells (human T cell leukemia) (Fig. 6a). It also bound to T-LAK cells (Fig. 6b), but bound very little to PBMCs (Fig. 6c). IL-21 co-stimulated anti-CD3-activated thymocytes and mature T cells [1]. Thus, our results show that IL-21R was also expressed on T-LAK cells stimulated by CD3. This suggests that the refolded rIL-21 may be suitable as an enhanced molecule in adoptive immunotherapy using T-LAK cells. The binding activity of the refolded protein was retained even after incubation for 1 week at 37°C (data not shown).

3.5. Proliferation assay of PBMCs and T-LAK cells

The rIL-21 did not induce the proliferation of PBMCs at any dose from 0.1 to 100 ng/ml (Fig. 7). On the other hand, it effectively stimulated the proliferation of T-LAK cells at doses from 1 to 100 ng/ml (Fig. 7). There was no remarkable difference in the concentration of the rIL-21 over the range (data not shown). These results reveal that rIL-21 has a significant effect on the proliferation of T-LAK cells.

3.6. Growth inhibition assay of TFK-1 cells mediated by T-LAK cells and PBMCs

rIL-21 strongly enhanced the inhibition by T-LAK of TFK-1 cells at effector:target (E:T) ratios of 5:1 and 10:1 (Fig. 8). No enhanced cytotoxic effect was found when PBMCs were used as effector cells and when only rIL-21 was used without effector cells (data not shown). The effect of rIL-21 on TFK-1 cells was saturated at nanogram concentrations.

3.7. rIL-21-mediated enhanced cytotoxicity against other cell lines

We assayed the inhibition by rIL-21 of two other cell lines: OBA-LK1 and DLD-1. The rIL-21 gave the same inhibition of both cell lines as it did of TFK-1 cells, although the sensitivity for the T-LAK cells was different (Fig. 9a, b).

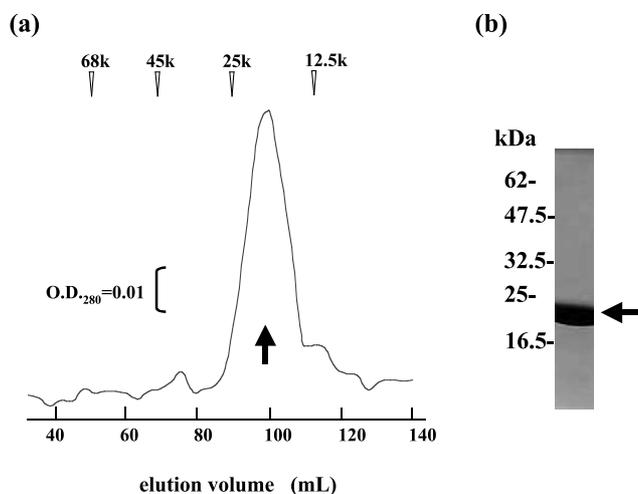


Fig. 4. Gel filtration of refolded rIL-21. (a) Profile of gel filtration. Refolded rIL-21 was subjected to gel filtration on Superdex-75 (Amersham) (2.6×60 cm) connected to an FPLC system, pre-equilibrated with PBS. 10 ml of refolded rIL-21 adjusted to 0.2 mg/ml was subjected to gel filtration at a flow rate of 3 ml/min. (b) SDS-PAGE of the eluted fraction.

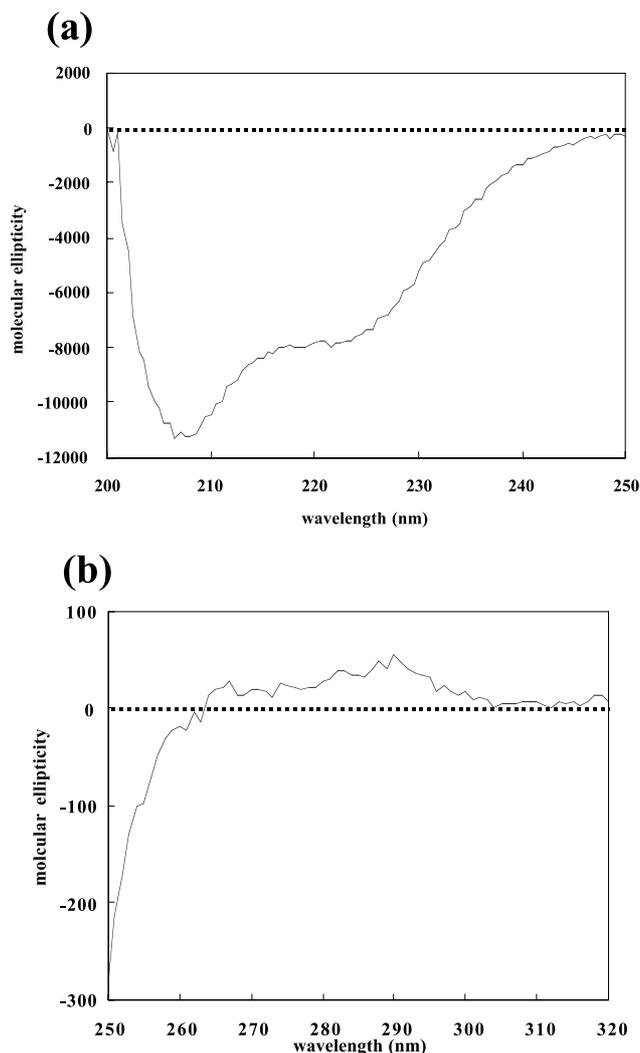


Fig. 5. Circular dichroism spectra of refolded rIL-21 from disulfide-shuffling system in the region of 200–250 nm (a) and 250–320 nm (b).

4. Discussion

Interleukin-21 is a recently discovered IL-2-like cytokine that upregulates T cells under CD3 stimulation [1,2]. Here we report large-scale preparation of *E. coli*-expressed rIL-21 in a high-yield refolding process. The yield of the protein using conventional refolding method (i.e. dilution) has been less than 50% (data not shown). On the other hand, the yield of recombinant IL-21 refolded from insoluble inclusion bodies overexpressed in *E. coli* was > 200 mg/L. The His-tagged rIL-21 has a refolding efficiency, physical stability, and biological activity identical to the rIL-21 without the His-tag (data not shown) and, thus, here we report the refolding and functional characterization of His-tagged rIL-21.

If rIL-21 is a type I cytokine as has been proposed [1], two disulfide bonds should exist. Disulfide bond formation is a critical step in the correct folding of proteins. We have recently reported a high-yield refolding process of single-chain antibody Fv fragments and other proteins with immunoglobulin folds [10]. Our method, however, gave a relatively low efficiency of rIL-21 refolding of 57%. The use of disulfide

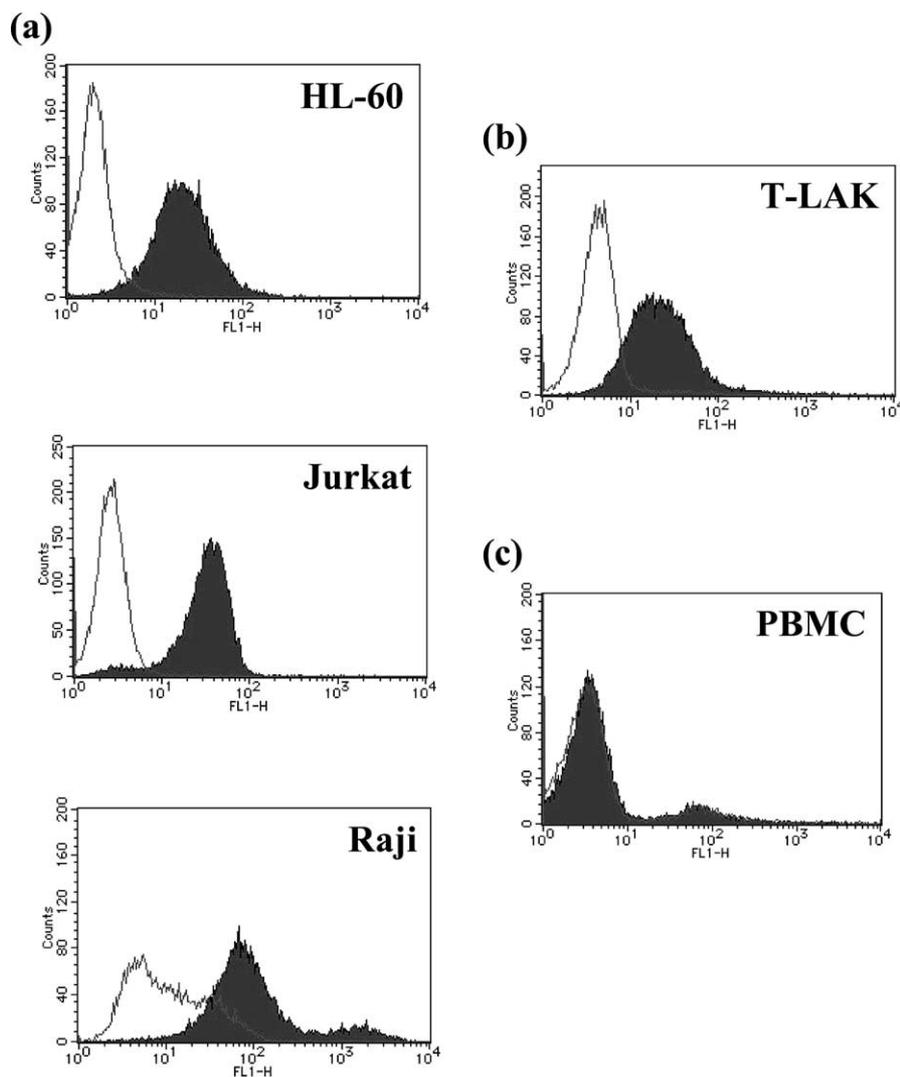


Fig. 6. Flow cytometry analysis of rIL-21 in (a) cell lines, (b) T-LAK cells, and (c) PBMCs. Shaded areas: cells incubated with rIL-21. After incubation, mouse anti-c-myc mAb 9E10 was added, followed by staining with FITC-conjugated goat anti-mouse IgG. Unshaded areas: cells incubated without rIL-21.

shuffling increased the refolding efficiency to 90%, indicating that refolding efficiency depends on protein folding and especially on the condition of the intramolecular disulfide bonds. The binding activity of the refolded protein was maintained and no aggregation was observed even after incubation for 1 week at 37°C (data not shown), suggesting the stable folding of the rIL-21. Surprisingly, the protein could be successfully refolded even at a relatively high concentration ($\sim 20 \mu\text{M}$; data not shown) with a high yield ($\sim 80\%$), suggesting that the correct formation of disulfide bonds at the appropriate refolding stage may be critical for refolding the protein with high yield. Note that the dialysis method developed for refolding of immunoglobulin-like proteins can be used for α -helical proteins if some parameters (e.g. concentration of redox solution) are correctly adjusted.

Some cytokines, for instance IL-2, IL-4, and IL-6, have been expressed in *E. coli* and prepared from insoluble inclusion bodies by refolding. In each case the proteins were functional and identical to ones purified from mammalian cells [19–22]. In addition, refolding increased the final yield by more than 100 times the yield of the soluble fraction (e.g.

periplasmic fraction) in *E. coli* [11]. However, a relatively low refolding efficiency, such as that of IL-6, which consists of 4-helix bundles with 2-disulfide bonds, is sometimes a problem, especially in large-scale preparation for therapeutic uses [23]. Therefore, our method could improve the preparation of cytokines in an *E. coli* expression system.

IL-2 has been used as a modulator in adoptive immunotherapy in many studies. However, its strong side effects have limited its use [24–26]. Our results show that rIL-21 has no effect on the proliferation of PBMCs and the cytotoxicity for tumor cell line using PBMCs (as a substitute of the effector cell for T-LAK cells) and using only rIL-21 without effector cells. This result provides strong support for the previous suggestion that IL-21 is a co-stimulatory molecule [1]. When injected into the bloodstream, fewer side effects might be expected than from IL-2. IL-21 promotes antigen-specific T cell activation [27]. In addition, as we found, the refolded protein seems to be stable under long-term incubation at 37°C. Therefore, human IL-21 may be a potent candidate as a co-stimulator.

In conclusion, *E. coli*-expressed human rIL-21 can be re-

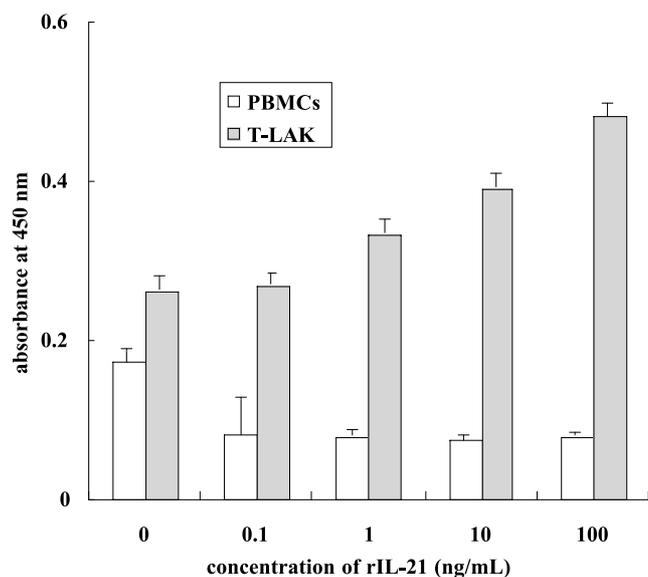


Fig. 7. Proliferation assay of PBMCs and T-LAK cells (72-h BrdU incorporation test). Freshly isolated PBMCs or T-LAK were incubated for 72 h with indicated doses of rIL-21. BrdU was then added to the culture. Incorporated BrdU was measured by the cell proliferation ELISA system (see details in Section 2). The columns show the results (mean values, with indicating S.D.) of triplicate determinations.

folded with high yield by using a disulfide-shuffling dialysis method. The prepared protein could recognize IL-21R-positive cell lines and was effective at promoting the proliferation of T-LAK cells. Growth inhibition assay showed that enhanced T-LAK killer activity against human bile duct carcinoma depended on concentration, indicating that IL-21 enhances cytotoxicity. Our recombinant IL-21 may help researchers discover new functions and effects of the cytokine.

Acknowledgements: This work was supported in part by Grants-in-Aid (R.A., K.T., and I.K.) from the Japan Society for the Promotion of Science and the Ministry of Education, Science, Sports, and Culture of Japan. Support was also provided through the Proposal-Based

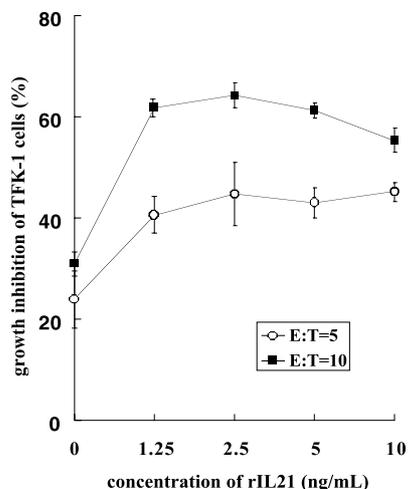


Fig. 8. Growth inhibition assay of human bile duct carcinoma TFK-1. Growth inhibition indices were determined by a 48-h MTS assay, in which rIL-21 and T-LAK cells (effectors) were added to TFK-1 cells (targets) at an effector:target ratio of 5:1 (○) or 10:1 (■). Data are mean values from at least three determinations.

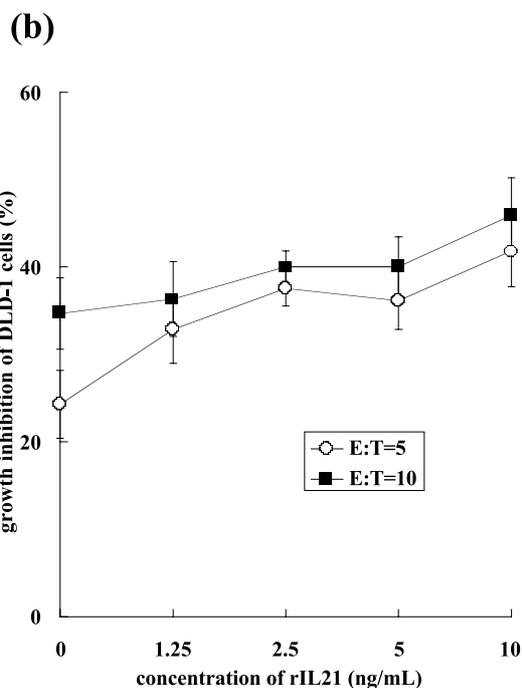
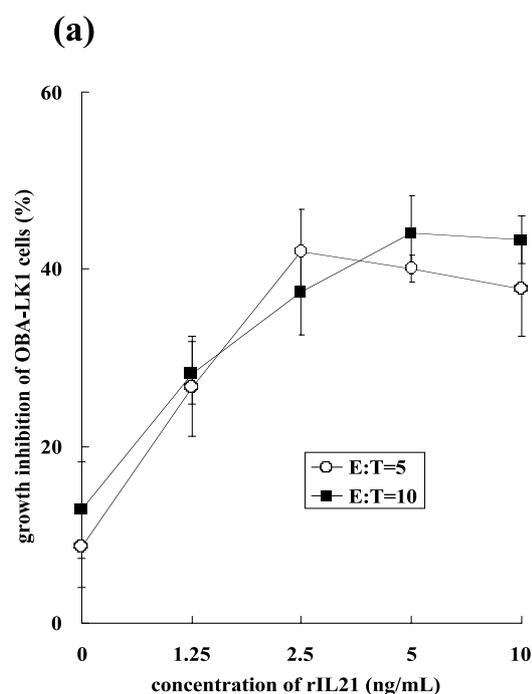


Fig. 9. Growth inhibition assay of (a) human lung carcinoma OBA-LK1 and (b) human colon cancer DLD-1. Growth inhibition indices were determined by a 48-h MTS assay, in which rIL-21 and T-LAK cells (effectors) were added to target cells at an E:T ratio of 5:1 (○) or 10:1 (■). Data are mean values from at least three determinations.

R and D Promotion Program and the Industrial Technology Research Grant Program from the New Energy and Industrial Technology Development Organization (NEDO) of Japan.

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