

Secretion of Biologically Active Human Interleukin-2 and Interleukin-4 from Genetically Modified Tobacco Cells in Suspension Culture¹

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Biologically active human interleukin-2 (IL-2) and IL-4, key lymphokines involved in immune regulation, were produced and secreted into the medium by genetically modified *Nicotiana tabacum* cells grown in suspension culture. Secretion through the plasma membrane and cell wall into the medium was facilitated by the natural mammalian leader sequences. IL-2 and IL-4 were detected in the medium at concentrations of 0.10 and 0.18 $\mu\text{g}/\text{mL}$, respectively, although higher levels were detected within the lymphokine-producing cells ($\approx 0.80 \mu\text{g}/\text{mL}$ for IL-2 and $\approx 0.28 \mu\text{g}/\text{mL}$ for IL-4). By Western blot, IL-4 was found to be secreted as two small polypeptides with molecular masses of approximately 18–20 kDa. The biological activity of IL-2 was determined by cell proliferation of the IL-2-dependent murine CTLL-2 cell line, while that of IL-4 was determined by cell proliferation of the CTLL-2 cell line [CT.h4S] which was stably transfected with the human IL-4 receptor. These findings indicate that plant suspension culture can be used to produce and secrete into the medium a variety of biologically active mammalian proteins that are of clinical and diagnostic relevance. © 1998 Academic Press

Key Words: genetic engineering; cultured tobacco cells; interleukin 2; interleukin 4.

The ability to produce mammalian proteins in whole plants and in cultured plant cell lines has led to an

increasing awareness of the potential value of transgenic plant systems for the inexpensive production of high-quality mammalian proteins for pharmaceutical and diagnostic purposes. Many of the early studies examining the feasibility of using transgenic whole plants to produce mammalian proteins focused on the expression of monoclonal antibodies in various tissues, especially in leaves (reviewed in 1). A few attempts were also made to produce other mammalian peptides and proteins in both intact plants and transgenic plant cells growing in culture. Among these was the expression in intact plants of the neuropeptide Leu-enkephalin as a fusion protein with the seed storage proteins, the 2 S albumins, from oilseed rape (2) and the expression of human serum albumin (HSA) in plant suspension cultures (3). In the latter case the HSA protein was demonstrated to be secreted by the cultured plant cells into the medium. Although the HSA was found to be correctly processed and indistinguishable from the authentic human protein (indicating that in plants posttranslational processing of proteins is more similar to that of mammalian cells than it is to that of bacteria) (3), the lack of a measurable biological function for HSA left unanswered the question of whether or not plants could produce and secrete biologically active mammalian proteins. This question was finally answered in the affirmative with the expression of human erythropoietin in cultured tobacco cells where the transgenic human protein was demonstrated to be biologically active in *in vitro* assays (4). However, rather than being secreted freely into the culture medium, the vast majority of the erythropoietin protein was found to be associated with the cell wall of the cultured tobacco cells (5).

The primary objective of the present study was,

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therefore, to further evaluate the ability of transgenic plant cell culture systems to produce and secrete biologically active mammalian proteins. For these experiments we chose to express the human cytokines IL-2³ and IL-4, which are ligands for membrane-bound receptors and which have small molecular masses, ≈ 15 and ≈ 19 kDa, respectively (6–8). These proteins were selected for study because we wished to examine the ability of cultured plant cells to produce secreted mammalian proteins whose biological activity is completely dependent on the proper folding and disulfide bond formation necessary for receptor binding to occur (9, 10). Human IL-2 is synthesized as a polypeptide of 153 amino acid residues (9). The first 20 amino acids represent a signal sequence that is cleaved to produce mature IL-2. Naturally occurring human IL-2 is a glycoprotein with varying amounts of glycosylation, giving a molecular weight range of 15,000–18,000. The mature protein contains three cysteine residues, two of which form a disulfide bond that is required for biological activity (9). IL-2 is normally synthesized and secreted by T helper cells and is important in driving the expansion of the antigen-specific cells (11). Because of the central role of IL-2 in the mediation of the immune response, IL-2 has been shown to have important diagnostic and therapeutic value (12).

The human IL-4 cDNA encodes for a precursor protein containing 153 amino acid residues (10). The signal peptide from the precursor is cleaved to yield a mature protein of 129 amino acid residues (13). It exhibits multiple immunomodulatory functions on a variety of cell types including T lymphocytes, B lymphocytes, monocytes, neutrophils, hematopoietic progenitors, fibroblasts, endothelial cells, and epithelial cells. Such a wide range of cellular targets suggests that it plays a central role in the modulation of immune and inflammatory responses (14, 15). For example, on T lymphocytes, IL-4 is an important modulator of the differentiation of T helper cells that regulate antibody production (16, 17). It is also important as a regulator for isotype switching involving the production of both IgE and IgG4 (14, 15). Thus, its role as a potential therapeutic reagent is also promising.

From our studies with IL-2 and IL-4, we found that both lymphokines were produced and secreted into the medium. Consistent with the findings for erythropoietin (5), both lymphokines exhibited biological activity when tested in *in vitro* assay systems. These results are important because they provide further impetus for

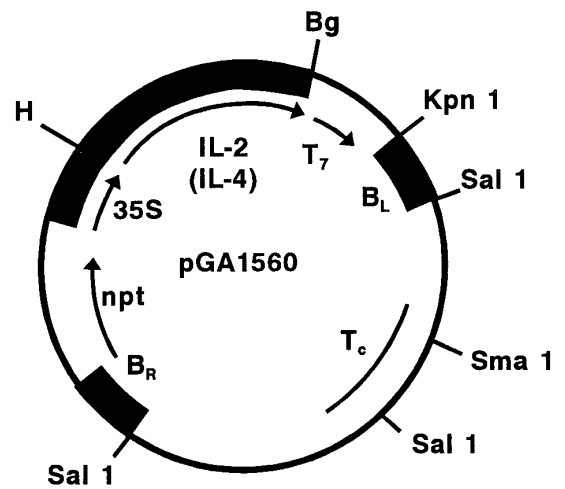


FIG. 1. Construction of plasmid pGA1560 as indicated carrying the cDNA for IL-2 (462 bp), which is a binary vector carrying a kanamycin resistance gene (*npt*) and a tetracycline resistance gene (*Tc*). The IL-2 cDNA was placed under the control of the CaMV 35S promoter and the T7 terminator. BL and BR are the left and the right borders of a T-DNA. A plasmid, designated pGA 1361, carrying the IL-4 cDNA was constructed using the same subcloning strategy.

the development of the plant cell suspension culture system as an inexpensive alternative for producing mammalian proteins for use as biopharmaceuticals in diagnosis and therapy.

MATERIALS AND METHODS

Construction of IL-2 and IL-4 expression vectors. The entire cDNA sequence of the human IL-2 gene, a gift of Dr. N. Holbrook (7), was inserted into the binary Ti plasmid pGA643. For the construction of the expression plasmid carrying IL-2, two new restriction sites flanking the coding region of the IL-2 cDNA were introduced by PCR to facilitate subcloning of the coding region into the Ti binary plasmid. The restriction sites were *Hind*III at the 5' end and *Bgl*II at the 3' end. The primer sequences to introduce these new restriction sites were primer 1, 5'-GGAAGCTTACAATGTACAGGATGCAAC-3' and primer 2, 5'-GGAGATCTATCAAGTTAGTGTG-3'. Three additional nucleotides immediately upstream of the ATG start code were included in primer 1 since this sequence matched the consensus DNA sequence near the start codon of the plant gene. The amplified fragment was blunt end cloned into the *Eco*RV site of pBluescript (SK⁻). From this plasmid, the *Hind*III and *Bgl*II fragment carrying the 462-bp IL-2 coding region was subcloned into the binary Ti plasmid vector pGA643. The resulting plasmid has been designated pGA1560. (Fig. 1). Likewise, the entire cDNA sequence of the human IL-4 gene, a gift of DNAX, Inc. (Palo Alto, CA) (8), was subcloned into the binary Ti plasmid pGA643 using the same PCR strategy. The primers used were primer 3, 5'-GGA-

³ Abbreviations used: IL-2, interleukin-2; IL-4, interleukin-4; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; BSA, bovine serum albumin; Fc, crystallizable fragment of antibody which is the constant region of the antibody heavy chains; PMSF, phenylmethylsulfonyl fluoride.

AGCTTGTAATGGGTCTCACCTCC-3' and primer 4, 5'-GGAGTCTCAGCTCGAACAACCTT-3'. Since the sequence immediately upstream of the start ATG codon of IL-4 did not match the consensus sequence of the plant gene, it was changed to GTA instead of TTA. Primer 3 included the *Hind*III site and primer 4 included the *Bgl*II site. As with IL-2, the IL-4 coding region was PCR amplified and inserted into the blunt end cloned into the *Eco*RV site of pBluescript (SK⁻). From this plasmid the *Hind*III/*Bgl*II fragment was subcloned into pGA643 and designated pGA1361 as indicated in Fig. 1. Each plasmid was transferred by the freeze method (18) into *Agrobacterium tumefaciens* LBA4404 that carried pAL4404 as a helper Ti plasmid (19).

Genetically modified plant cells. Genetically modified plant cells were generated as previously described (20). Briefly, *Nicotiana tabacum* (NT-1) suspension cells were cocultivated for 3 days with *A. tumefaciens* carrying the newly constructed binary Ti plasmid vector (18). The genetically modified cells were plated onto Murashige and Skoog (MS) agar medium containing 50 µg/ml kanamycin for the selection of transformants (18). Suspension cultures were developed by subculturing the transformed callus in a liquid medium containing 4.3 mg/mL MS salt supplemented with 3% sucrose, 0.18 mg/mL KH₂PO₄, 0.1 mg/mL inositol, 1 µg/mL thiamine hydrochloride, 0.2 µg/mL 2,4-dichlorophenoxyacetic acid, 100 µg/mL cefotaxime, and 50 µg/mL kanamycin. The genetically modified cells were cultivated in 250-mL Erlenmeyer flasks (working volume: 60 mL at 29°C with the speed of 150 rpm on an orbital shaker). The suspension cell lines were subcultured weekly with a 5% inoculum of 7-day-old cells.

ELISA analysis. For sample preparation, 500 µL of buffer (0.15 M NaCl, 50 mM Tris, pH 7.4, 0.25% NP-40) and 18 µL PMSF (0.01 g/mL) were added to 0.05 g of wet cells. The suspension was sonicated on ice for three 8-s bursts and then centrifuged at 15,000g for 5 min. The supernatant was collected for the ELISA assay. For detection and quantification of IL-2, an ELISA kit (Becton-Dickinson) was used according to the manufacturer's instructions. For detection and quantification of IL-4, an antigen capture ELISA was developed. All steps were carried out at room temperature. In brief, 96-well plates were coated with 50 µL of a 1:500 dilution of a mouse anti-human IL-4 monoclonal antibody (Pharmingen). Plates were blocked with phosphate-buffered saline made 10% in fetal calf serum and 0.05% Tween 20. Samples (100 µL) were added to the wells and incubated for 4 h. After the wells were washed, 100 µL of biotinylated rat anti-human IL-4 monoclonal antibody (Pharmingen) at a 1:500 dilution was added to each well and incubated for 45 min. After the wells were washed, 100 µL of avidin-conjugated peroxidase (Sigma) at a 1:400 dilution of a 2.0 mg/mL

solution was added to each well and incubated for an additional 30 min. After the wells were washed, 100 µL of 0.55 mM 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (Sigma) was added (21) and incubated for 10 min before the absorbance was read at 405 nm. Sample concentrations were determined by comparison to a standard curve of recombinant human IL-4.

Western blot analysis. Western blot analysis was carried out with rat anti-human IL-4 monoclonal antibody-biotin (Pharmingen) used at 1:2000 as the primary antibody and avidin-conjugated peroxidase (Sigma) used at 1:20,000 as the secondary reagent as described by Coligan *et al.* (22). The immune complexes were detected by enhanced chemiluminescence (Amersham).

Biological activity assays: IL-2. The IL-2 contained intracellularly or secreted into the medium was quantified on murine IL-2-dependent cell line, CTLL-2, using a modified biological assay for human IL-2 (23, 24). In brief, cytokine standard and sample dilutions were added to quadruplicate wells of Costar 96-well flat-bottomed microculture plates in a final volume of 100 µl per well. CTLL-2 indicator cells were washed by centrifugation three times in phosphate-buffered saline made 1% in fetal bovine serum (FBS) to remove IL-2 and then resuspended to a final concentration of 2×10^5 viable cells per milliliter in complete medium, RPMI 1640 with 25 mM Hepes buffer supplemented with 10% final concentration FBS, L-glutamine, gentamicin, and 0.05 mM 2-mercaptoethanol. The suspension (100 µL) was added to all wells. The plates were incubated for about 20 h in a humidified 5% CO₂ atmosphere at 37°C. Four hours prior to termination of the assay, tritiated thymidine (1 µCi/well) was added. At termination, the cells were harvested and thymidine incorporation was determined by scintillation counting. One unit of activity is defined as the amount of IL-2 that induces 50% of maximal thymidine incorporation.

Biological activity assays: IL-4. The intracellular and extracellular levels of biologically active human IL-4 were quantitated using a modified biological assay for human IL-4 (24) with the cell line CT.h4S (provided by Dr. W. Paul, NIAID, NIH, Bethesda, MD), a derivative of the CTLL cell line which was transfected with the human IL-4 receptor and selected for dependence on IL-4. The protocol for measuring human IL-4 is similar to the assay for IL-2 with the exception that the cells are exquisitely sensitive to the absence of IL-4 and the initial incubation period is 24 h longer (22). Briefly, 16–20 h prior to the assay, the cells were subcultured to ensure that they were in log phase of growth. Immediately before using, the cells were washed to remove IL-4 and resuspended in fresh medium deficient in IL-4. The cells were added to samples and standard prepared 96-well flat-bottomed plates. The CT.h4S cells were cultured for 40 h at 37°C before

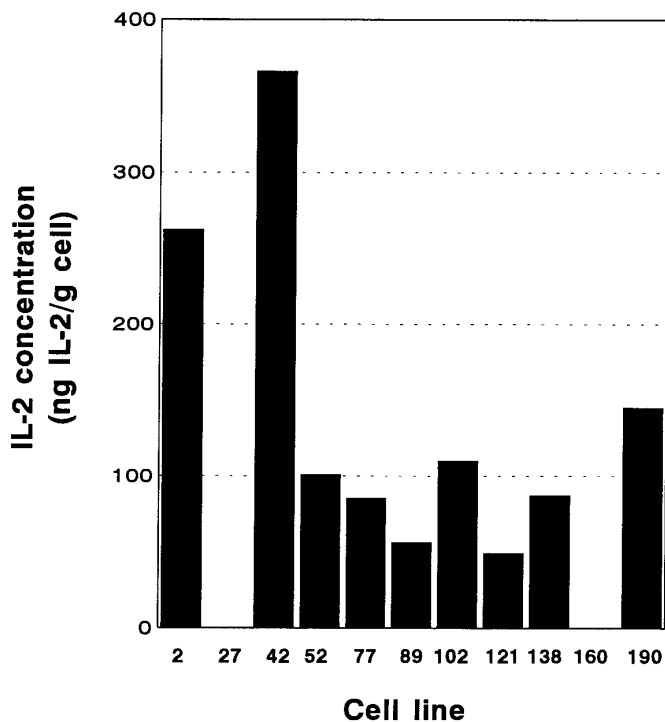


FIG. 2. IL-2 production as measured by ELISA of lysates produced from transgenic tobacco cells grown as calli. Eleven separate calli were selected for IL-2 determination in this experiment.

the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was added. The plates were further incubated for 24 h at 37°C. After incubation, the absorbance of each well was measured at 492 nm. Assay specificity was assessed by analysis of replicate supernatants in the presence of anti-IL-4 neutralizing antibody (40 ng/mL, Sterling Winthrop Inc.). CT.hS4 did not respond to concentrations of rhIL-2 below 666 pg/ml (unpublished data). All samples were evaluated at least twice, with the concentration of cytokine in each supernatant calculated from a minimum of three points falling on the linear portion of an internal standard generated with rhIL-4 (sp act 1.8×10^7 U/mg, Sterling Winthrop Inc.). Standard deviations for this assay were routinely <5%.

RESULTS

Generation of Transgenic Tobacco Cells

For IL-2-expressing cells, over 200 independently transformed tobacco calli were initially selected in the presence of kanamycin and maintained on agar medium. Eleven of these were randomly chosen and tested for IL-2 production by ELISA. Figure 2 demonstrates the variable expression level found for these selected calli with 9 of the 11 clones producing IL-2. Figure 3 demonstrates the time course for IL-2 production in batch suspension culture of the higher IL-2-producing

calli designated clone 2. In the batch culture method, medium is inoculated with a 5% suspension of plant cell culture and IL-2 production is followed over a period of 7 days in culture. Intracellular levels of IL-2 are compared with secreted levels of IL-2. As can be seen in this example, the highest level of IL-2 production, when combining both intracellular and secreted IL-2 (≈ 90 ng IL-2/mL total), is on day 5 with the intracellular level of IL-2 being higher (sevenfold) than that detected in the medium. It should also be noted that extensive screening for high-IL-2-producing calli was not conducted at this time due to the expense of the screening reagents.

For identification of IL-4-producing cells, over 200 independently transformed calli were also selected in the presence of kanamycin and subsequently maintained on agar medium. Of 100 calli tested, 99 were found to express IL-4. As shown in Fig. 4, 9 clones of calli randomly selected were analyzed for IL-4 production and of these, 8 were found to be positive for IL-4. In terms of IL-4 production, the overall level was much higher (1100 ng/g calli) than that found for IL-2 (366 ng/g calli) due in part to the availability of reagents to screen larger numbers of IL-4-transformed calli. Comparing the expression level of IL-4 from clone 142 adapted to suspension culture, Fig. 5 shows that on day 5, the intracellular IL-4 level was ≈ 0.275 mg/L

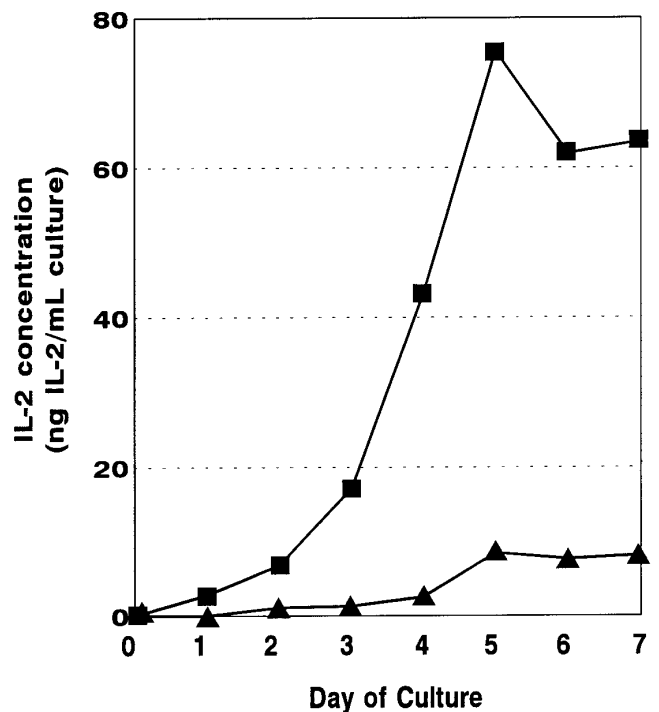


FIG. 3. Time course of IL-2 production in batch culture of clone 2 as detected by ELISA. The culture was monitored over a 7-day culture. Squares represent IL-2 detected intracellularly. Diamonds represent IL-2 secreted into the medium.

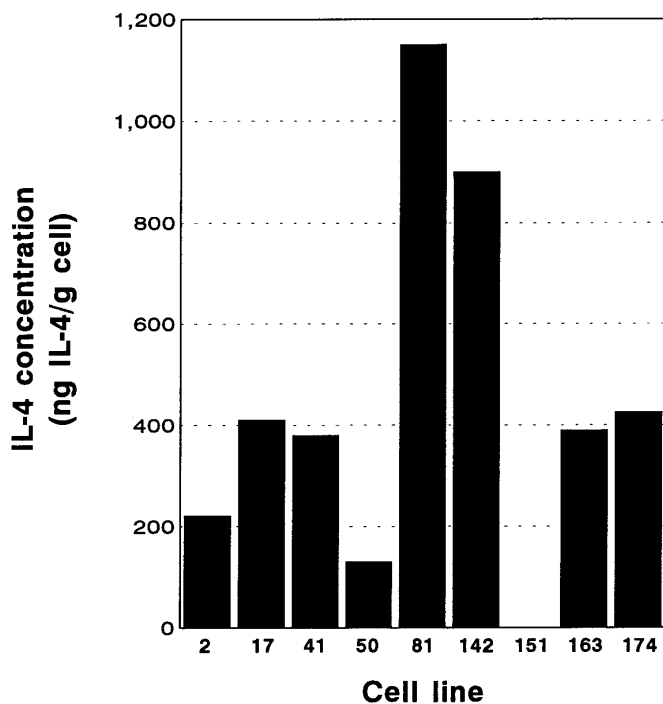


FIG. 4. IL-4 production as measured by ELISA of lysates produced from transgenic tobacco cells grown as calli. Nine individual calli were assayed for IL-4 production in this experiment.

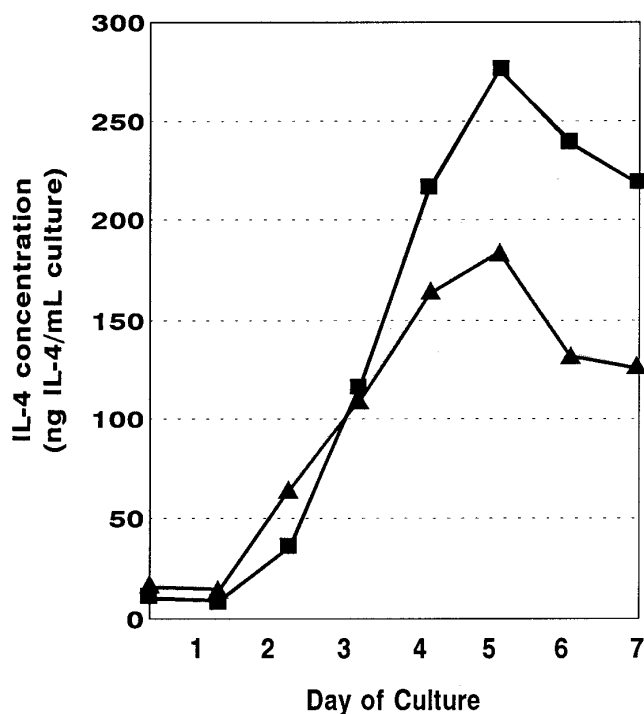


FIG. 5. Time course of IL-4 production in batch culture of clone 142 as detected by ELISA. The culture was monitored over a 7-day culture. Squares represent IL-2 detected intracellularly. Diamonds represent IL-2 secreted into the medium.

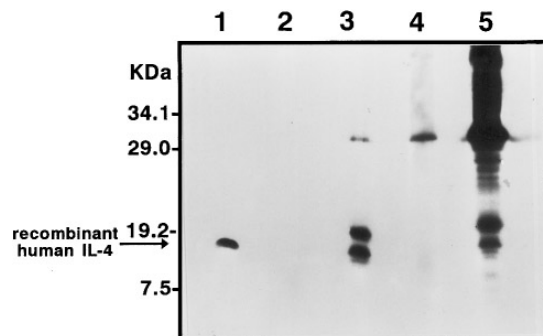


FIG. 6. Western blot analysis of recombinant human IL-4 produced by clone 17 grown in batch cell culture. Secreted IL-4 was TCA precipitated from 1 ml of medium from 6-day-old cultures (lane 3) or from lysates of cells contained in 1 ml of the same 6-day-old cell cultures (lane 5). Intracellular (lane 4) and extracellular (lane 2) samples of control cells (NT-1) were prepared in a manner similar to samples from clone 17. The positive control was 150 ng of recombinant human IL-4 (lane 1).

while the secreted level was ≈ 0.180 mg/L for a total of ≈ 0.455 mg/L of culture.

Detection of Lymphokine by Western Blot

With the finding that both IL-2 and IL-4 could be detected in culture medium by ELISA, it was of interest to determine whether the secreted lymphokines possessed the same characteristics as the intracellularly produced lymphokines and the commercially available recombinant human lymphokines. Unfortunately, evaluation of several commercially available antibodies to human IL-2 did not yield an antibody that could be used in Western blot analysis. On the other hand, an excellent antibody was found for IL-4. Therefore, to identify the presence of IL-4 in the medium, 1.0 mL of suspension culture was separated into medium and cells. The cells were resuspended in 1.0 mL of medium and lysed by sonication. Each sample was TCA precipitated and analyzed by Western blot. As shown in Fig. 6, two bands running close together at approximately 18 kDa were present in both the medium from clone 17 and the intracellular contents of clone 17. This corresponded to the molecular mass exhibited by the commercially available recombinant IL-4. It is not clear whether the two bands represent two different glycosylated forms of the IL-4 or a processed and incompletely processed form of IL-4.

Biological Activity

Although IL-2 and IL-4 could both be detected by ELISA and IL-4 could be visualized by Western blot, it was important to determine whether the secreted proteins were biologically active. The most sensitive biological assays available are those which assess the ability of the lymphokine to induce proliferation of lym-

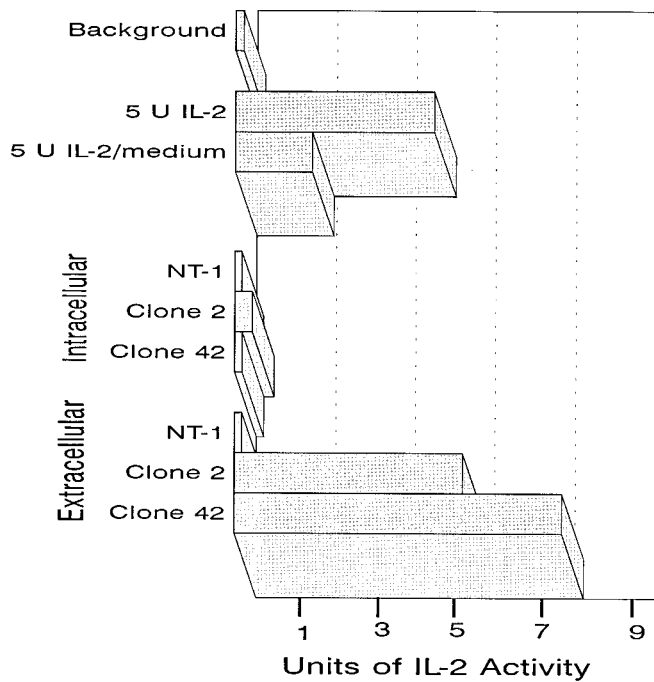


FIG. 7. Biological activity of human IL-2 secreted into the medium from clone 2 and 42 transgenic tobacco cells. Five units of recombinant human IL-2 was used as a standard in the biological assay as described under Materials and Methods. Aliquots (50 μ l) from 6-day-old cultures were analyzed in the assay.

phocytes that require the lymphokine for survival. Samples were tested as either aliquots of medium directly or aliquots of sonicated cell lysates. As shown in Fig. 7, 50- μ l aliquots of medium from 6-day cultures from two different clones of IL-2-producing cells lines (2 and 42) had substantial IL-2 activity, 5.2 and 8.2 ng/mL, respectively, secreted in the medium. In fact, this activity closely corresponded to the amount of secreted protein, indicating that a major portion of the secreted IL-2 was biologically active. The amount of biologically active material may have been even higher as the medium alone was found to be somewhat inhibitory to the mouse indicator cells in the proliferation assay. Although almost 10-fold more IL-2 protein was detected intracellularly by ELISA, the biological assay suggests that very little activity existed inside the cells. However, this observation may be misleading and is more likely attributed to the inhibitory nature of the crude cell lysates on the indicator cells (data not shown).

The secreted IL-4 also exhibited strong biological activity. However, in the case of IL-4, only one-fourth of the biological activity was accounted for relative to that expected for the amount of protein being produced. For clone 17 the IL-4 biological activity assay indicated that 13.9 ± 4.3 ng/mL of active IL-4 was being secreted into the medium. From the ELISA it appeared that closer to 54.4 ng/mL was being produced for this clone. Since

the IL-4 was assayed directly in the culture medium without any purification, it is possible that the activity was much higher than observed.

DISCUSSION

We have made two important findings for tobacco cells carrying the transgenes for human IL-2 and IL-4 grown in suspension culture. The first finding is that the cells not only produce these lymphokines, they also secrete them into the medium through both the plasma membrane and the cell wall. The second finding is that the secreted lymphokines have biological activity. Possessing biological activity appears to be a consistent observation for plant-produced recombinant proteins. In fact, in every case where it has been possible to assess biological activity of recombinant proteins produced by plants, the finding of biological activity has been confirmed. Such recombinant proteins include antibodies (1, 25–29), human interferon (30), lysozyme (31, 32), and human erythropoietin (4, 5). In addition, reports of transgenic proteins being secreted from plant cells have been accumulating over the years, further suggesting plant cells as a potential alternate source of recombinant proteins (4, 5, 20, 28, 31–33). The advantage that secretion of recombinant protein would have over nonsecreted proteins is the reduced number of steps required in the purification process of the transgenic protein. This is because plant cell culture medium is relatively simple, consisting primarily of water with a few salts, sugars, and vitamins (34).

The possibility of using plants to produce economically important proteins has been under investigation for almost a decade. Production of transgenic proteins in whole plants and in plant cells in suspension culture has been well documented but unambiguous demonstration of secretion of a biologically active transgenic protein from plant cells has only recently been shown (20, 28, 33). The first demonstration of a transgenic protein being secreted from plant cells was with human serum albumin at a level of 0.25 μ g/mg of plant protein (3). Unfortunately because albumin lacks a measurable enzymatic or biological activity, it was not possible to assess the functional capacity of the plant-produced albumin. Therefore, possibly the first demonstration that a secreted recombinant protein from plant cells had biological function was with hen egg lysozyme (31). It was noticed that bacteria were lysed in the agar surrounding the transgenic calli. This suggested that the lysozyme was being secreted from the calli, which was a reasonable speculation since the lysozyme cDNA used for generating the transgenic plant cells included a leader sequence. It was becoming clear that a leader sequence of either plant or animal origin could facilitate the targeting of the transgenic protein to the endoplasmic reticulum and eventually directed it through the plasma membrane (25, 29). The question of

whether or not a functional transgenic protein was being secreted across the plant cell wall, however, was not completely clear in these experiments because the detectable level of lysozyme secreted into the agar was so low, it was not measured and there was the possibility that the biological activity observed was a result of plant cell lysis (3, 26, 27, 29, 30).

One of the major questions about generating recombinant proteins in the plant system has been whether they would be processed appropriately and, therefore, have biological activity. Our first attempt to express a functional transgenic protein involved a mouse monoclonal heavy chain (20). In this case, biological activity was detected by the ability of the antibody heavy chain to bind its cognate antigen, *p*-azophenylarsonate, and by its ability to bind protein G via the Fc region of the heavy chain. Expression of IL-2 and IL-4 was undertaken in the present study because it was of interest to determine whether a protein could be expressed and secreted from plant cells whose biological activity is critically dependent upon it being capable of binding and activating its receptor. For IL-2 we found amounts of biological activity that closely corresponded to the amount of protein being produced. For IL-4, however, we found only about one-fourth of the activity expected for the amount of protein detected. On Western blot (Fig. 7) two bands running near 19 kDa were found. It is not known whether there is a connection between the two bands and the level of activity expected. Furthermore, the difference between the two bands may be due to differences in glycosylation or perhaps due to an unprocessed IL-4 which still retains the signal sequence. However, it should be kept in mind that naturally occurring human IL-4 does exist in multiple molecular mass forms between 15 and 19 kDa (8). Recombinant IL-4 produced in a baculovirus expression system has an approximate molecular mass of 14 to 15 kDa, whereas IL-4 produced in yeast is quite heterogeneous in molecular mass with some forms exhibiting sizes of ≈ 50 kDa. As with many other glycoprotein growth factors, IL-4 that has been deglycosylated by digestion with endoglycosidase F has full biologic activity *in vitro*, as does recombinant IL-4 produced in *Escherichia coli*. Therefore, further biochemical characterization of the secreted IL-4 remains to be carried out to determine the extent of posttranslational modification (i.e., glycosylation) carried out by the plant cells.

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