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ANALYSIS OF IL-2 PRODUCTION IN HOOKWORM INFECTED HAMSTERS USING GENERATED POLYCLONAL ANTIBODIES

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Abstract

The aim of the study was cloning and analysis of the entire coding sequence of hamster IL-2 by the method of RACE-PCR, its expression in *Escherichia coli* cells, and production of IL-2 specific antibodies. These antibodies were used to determine *in vitro* IL-2 production by cells derived from the spleen and mesenteric lymph nodes of *Ancylostoma ceylanicum* infected hamsters. The highest concentration of IL-2 was noted in supernatants from cell cultures coming from the oldest, most resistant hamsters.

Key words: hamster, hookworm, antibodies, IL-2, RACE-PCR.

cDNA of human interleukin 2 (IL-2) was the first among cytokine cDNAs to be cloned in 1983 (11, 35). It is a monomeric, secreted glycoprotein with a molecular mass of 15 kDa. Its crystal structure was solved in 1992 (5). Its structure consists of four α-helices folded in a configuration typical of the type I cytokine family.

IL-2 was initially named T-cell growth factor (TCGF) as its first described function was potent enhancement of *in vitro* proliferation and differentiation of T-cells (19, 31). Subsequent studies revealed many other immune enhancing functions of this cytokine such as supporting of proliferation and survival of T cells, differentiation of naive T cells into effector and memory cells, and ability to overcome the proliferation block of anergic cells (reviewed in 3). IL-2 also plays an important role in immunoregulation. It promotes activation-induced cell death (AICD) of T cells (25, 34) and stimulates the generation and homeostasis of regulatory T cells (T_{REG}), which are responsible for immunological self-tolerance and regulation of immune responses (10, 14, 23).

The Syrian golden hamster (*Mesocricetus auratus*) is uniquely susceptible to a variety of viral, bacterial, and protozoan pathogens, such as West Nile virus (36), *Leishmania* sp. (28), or *Leptospira* (20). Hamsters are used in studies concerning hookworm infection (2, 7), and also pancreatic and oral cancer (26, 33). The molecular basis for this high level of susceptibility is unknown and immunological studies related to this model have been limited by the lack of available reagents.

So far cytokine expression analysis is possible only on mRNA level as fragments of hamster cytokine cDNAs have been cloned by Melby *et al*. (28). Recently, we have reported production of chicken polyclonal antibodies recognising hamster cytokines and their possible use in determination of cytokine concentration (12). The aim of this study was cloning of full length cDNA encoding another hamster cytokine, IL-2, sequence analysis, and its expression in *E. coli* cells. Purification of recombinant IL-2 was performed in order to immunise chickens and produce IL-2 specific polyclonal antibodies. Generated antibodies were then used to estimate *in vitro* production of IL-2 by spleen and mesenteric lymph node cells originating from hookworm infected hamsters.

Material and Methods

Cloning of IL-2 full length cDNA. RNA was extracted from hamster spleen with "Total RNA" kit (A&A Biotechnology, Poland) and then used as a template in a reverse transcription reaction with RevertAid H Minus M-MuLV reverse transcriptase (MBI Fermentas). cDNA encoding hamster IL-2 was amplified by RACE-PCR method, therefore cytosine nucleotides were added to the 5' end of the cDNA with the use of terminal deoxynucleotydil transferase (MBI Fermentas). Internal primers for amplification of cDNA were designed on the basis of partial cDNA of hamster IL-2, reported by Melby *et al*. (28) (Accession number AF046212). Primer sequences are shown in Table 1.

Amplification of the 5' end was performed with *Taq* polymerase (MBI Fermentas) in MJ research thermocycler under the following conditions: initial denaturation at 94ºC for 3 min, 40 cycles of denaturation at 94ºC for 30 s, annealing at 58ºC for 30 s, elongation at 72ºC for 30 s, and final elongation at 72ºC for 5 min. Conditions for 3' end amplification were the same except for annealing temperature, which was 53ºC. Amplified products were ligated into pBluescript SK II (+) vector and electrotransformed into *E. coli* DH5α cells. Single colonies were screened for recombinant plasmids, positive clones were confirmed by colony PCR and restriction analysis. Plasmids were then sequenced in both directions.

Sequence and analysis of IL-2 cDNA. The determined nucleotide and deduced amino acid sequences were compared with sequences encoding IL-2 from other species published in GenBank. With the use of needle programme sequence identities and similarities were determined (http://www.ebi.ac.uk/Tools/psa).

Molecular masses and pI were predicted using compute pI/Mw tool on Expasy server (www.expasy.org/tools/pi_tool.html), N- and O-glycosylation sites were predicted with NetOGlyc and NetNGlyc programmes (www.cbs.dtu.dk/services) (22) and signal sequences were predicted with SignalP programme (www.cbs.dtu.dk/services/SignalP) (6). Disulfide bonds were predicted with DiANNA 1.1 programme (http://clavius.bc.edu/~clotelab/DiANNA) (13).

Expression and purification of recombinant IL-2 (rIL-2) in *Escherichia coli* cells. Amplification of IL-2 cDNA was performed under the same conditions as cloning, except annealing temperature, which was 56ºC. Primers contained restriction sites for *Bam*H1 (IL-2F1) and *Sal*1 (IL-2R1), which enabled cloning amplified cDNA into pET 28 expression vector (Table 1). Recombinant plasmids were then electrotransformed into *E. coli* BL21 (DE3) cells. Transformed cells were cultured in LB medium until the late log phase and expression was induced by addition of 1 mM isopropyl thiogalactoside (MBI Fermentas). The cells were cultured for 3 h at 30°C, and then centrifuged (5,500 x g for 15 min). The pellet was resuspended in equilibration buffer (2.5 mM imidazole; 0.3 M NaCl; 50 mM Na_2HPO_3; 4 M urea, pH 8), disrupted by sonication, and incubated at 4°C for 12 h. The sample was then centrifuged (15,000 x g for 20 min), the supernatant was filtered through 0.45 µm pore filter, and applied on His-Select chromatography cartridge (Sigma). rIL-2 was then eluted from the column with elution buffer (0.5 M imidazole; 0.5 M NaCl; 20 mM Tris-HCl; 4 M urea, pH 8). The recombinant protein was further electroeluted from the polyacrylamide gel in order to eliminate remaining bacterial proteins. Fractions containing affinity purified rIL-2 were separated by SDS-PAGE, then gel slices containing rIL-2 were electroeluted in Bio-Rad Electro-Eluter according to manufacturer's instructions. The purity and specificity of rIL-2 was determined by SDS-PAGE. The concentration was defined using BCA Protein Assay Kit (Pierce).

Generation and analysis of IL-2 specific chicken polyclonal antibodies. Two 6-week-old Leghorn white chickens were immunised intramuscularly with 0.5 mg of IL-2 recombinant with Freund incomplete adjuvant as described previously (12). The specificity of antibodies was determined by Western blotting. Briefly, nitrocellulose membranes containing samples of rIL-2 and splenocyte culture supernatant were incubated with chicken IL-2 specific antibodies diluted 1:200 for 2 h, followed by rabbit anti-chicken IgY-HRP conjugated antibodies (1:3,000) (Sigma). Bands were visualised using SuperSignal West Pico Luminol/Enhancer Solution (Pierce) and chemiluminescence was detected by exposure of blots to autoradiography film (Kodak).

Hamsters. Three age groups of 4-week, 8-week, and 6-month-old male Syrian golden hamsters (*Mesocricetus auratus*) (n=5) were used. The animals were maintained under standard conditions according to local regulations and all experiments were approved by the local ethical committee. The hamsters were infected orally with 60 infective larvae (L3) of *A. ceylanicum*. Twenty-one days after the infection, the animals were euthanized. Adult worms were removed from the small intestine and counted.

Cell cultures. Spleen and mesenteric lymph nodes were removed from infected hamsters and single-cell suspensions were prepared and cultured as described before (12) with medium alone, ConA (Sigma) at a final concentration of 5 µg/mL, or *A. ceylanicum* somatic antigen at concentration of 30 µg/mL. The plates were incubated at 37°C in an atmosphere of 5% CO_2 for 72 h. Then cell-free supernatants were collected.

ELISA. IL-2 concentrations were determined using a direct ELISA. 96-well plates were coated with 100 µl of culture supernatants for 12 h at 4°C. The procedure was the same as reported previously (12), except that rIL-2 was used to prepare the standard curve, and IL-2 antibodies were used. The plates were developed with the TMB Substrate (Sigma) and read in MRX plate reader (Dynatech Laboratories) at 450 nm.

Statistics. Statistical significance of differences between groups was determined with non-parametric Mann-Whitney test. A value of $P<0.05$ was considered to be significant. Analysis was done using Statgraphics Plus 4.1 software.

Results

The full length cDNA of hamster IL-2 was cloned using RACE-PCR. The 5' and 3' ends were amplified and cloned into pBluescript SK II (+) vector, which was then sequenced in both directions. Overlapping fragments of both cDNA ends were found and the entire sequence was determined. The full length cDNA sequence consisting of 805 nucleotides was submitted to GenBank and assigned accession number EU729351.

Table 1
Nucleotide sequences of primers used for 5'RACE-PCR, 3'RACE-PCR and for cloning of IL-2 cDNA into pET 28 expression vector

		Primer sequence
5'RACE-PCR:	IL-2R:	5'CATCTGTTCAGAAATTCCACCA3'
	oligo$(dG)_{18}$:	5'AAGAATTCGGGGGGGGGGGGGGGGG3'
3'RACE-PCR	IL-2F:	5'GGATCCATCCTGTCTTGCACTGACGCT3'
	oligo$(dT)_{18}$:	5'AGAGAACTAGTTTTTTTTTTTTTTTT3'
Cloning into pET28	IL-2 F1:	5'GGATCCATCCTGTCTTGCACTGACGCT3'
	IL-2 R1:	5'GTCGACCATCTGTTCAGAAATTCCACCA3'

Table 2
Comparison of amino acid (AA) similarities and identities, and characteristics of IL-2 of: hamster (*Mesocricetus auratus*), deer mouse (*Peromyscus maniculatus*), cotton rat (*Sigmodon hispidus*), rat (*Rattus norvegicus*), Mongolian jird (*Meriones unguiculatus*), Rhesus monkey (*Macaca mulatta*), human (*Homo sapiens*), mouse (*Mus musculus*), and dog (*Canis lupus familiaris*)

Organism	GenBank accession number	AA Identity (%)	AA similarity (%)	Mature protein			Signal pepitide (AA)	O-Glyc sites	N-Glyc sites	Disulfide bonds
				AA	Mw(Da)	pI				
Mesocricetus auratus	ACE62917	-	-	135	15,517.86	5.63	20	0	0	1
Peromyscus maniculatus	AAP04419	85.2	89.6	>132*	14,931.11	5.55	20	0	0	1
Sigmodon hispidus	AAK94012	83.7	88.1	135	15,459.93	5.22	20	0	1	1
Rattus norvegicus	NP_446288	83	88.1	135	15,493.97	6.34	20	1	0	1
Meriones unguiculatus	CAA48679	82.2	91.9	135	15,496.89	5.38	20	1	0	1
Macaca mulatta	NM_001047130	73.3	83.7	134	15,475.05	6.35	20	0	0	1
Homo sapiens	NM_000586	73.3	83	133	15,418.01	7.05	20	0	0	1
Mus musculus	NP_032392	67.8	78.5	149	17,231.38	4.88	20	3	0	1
Canis lupus familiaris	AAA75360	65.4	79.4	135	15,536.68	4.91	20	0	1	1

* - only partial coding sequence is known

The full length sequence comprised the 5'untransribed region (5'UTR), the open reading frame (ORF) of 468 bp between positions 48 and 515, and the 3'UTR with a poly(A) tail. The amino acid sequence of hamster IL-2 was deduced. It consisted 155 residues with 20 aa long signal sequence. The predicted molecular mass of the mature protein was 15,517.86 Da and the pI was 5.63. No N- or O-glycosylation sites were found. One disulfide bond was predicted between cysteines at positions 58 and 106. Amino acid sequence was then aligned with sequences from other species. Results of IL-2 amino acid similarity and identity between species and comparison of protein characteristics are shown in Table 2.

cDNA encoding hamster IL-2 was cloned into pET 28 expression vector and the recombinant protein was produced in *E. coli* BL21 (DE3) cells. rIL-2 with molecular mass of about 18 kDa was purified under denaturating conditions on nickel chromatography column. SDS-PAGE analysis revealed that the sample contained remaining bacterial proteins (Fig. 1A). In order to obtain a homogeneous sample, rIL-2 was electroeluted from the polyacrylamide gel (Fig. 1B).

Leghorn white chickens were immunised with purified rIL-2. Western blot analysis of generated polyclonal IL-2 specific antibodies showed that they recognised recombinant IL-2 (Fig. 2A) and a protein of a molecular mass of about 30 kDa in splenocyte culture medium (Fig. 2B), which corresponded to dimeric form of IL-2.

Out of the three *A. ceylanicum* infected groups of hamsters, the youngest were the most susceptible to hookworm infection (42 ±7 worms), 8-week-old hamsters were infected with 35 ±18 worms, and the oldest ones had significantly the lowest number of worms (7 ±6). The concentration of IL-2 in cell culture supernatants also differed depending on the age of animals. Cells derived from the oldest hamsters produced the biggest amount of the cytokine independently of type of stimulation, but only after *A.ceylanicum* somatic antigen stimulation these differences were statistically significant (Fig. 3).

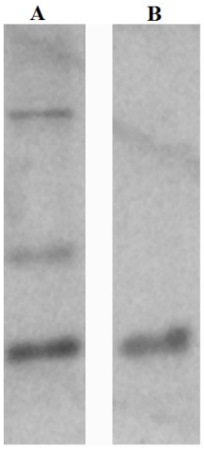

Fig. 1. SDS-PAGE analysis of rIL-2 after affinity purification on nickel column (A) and after additional electroelution from polyacrylamide gel (B).

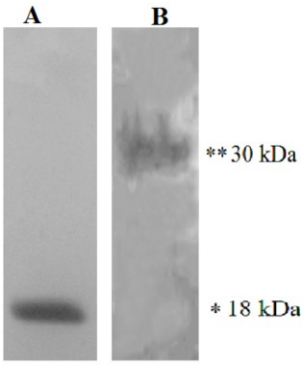

Fig. 2. Western blot analysis of chicken polyclonal antibody reactivity with purified rIL-2 (A) and native IL-2 in hamster splenocyte culture medium (B)

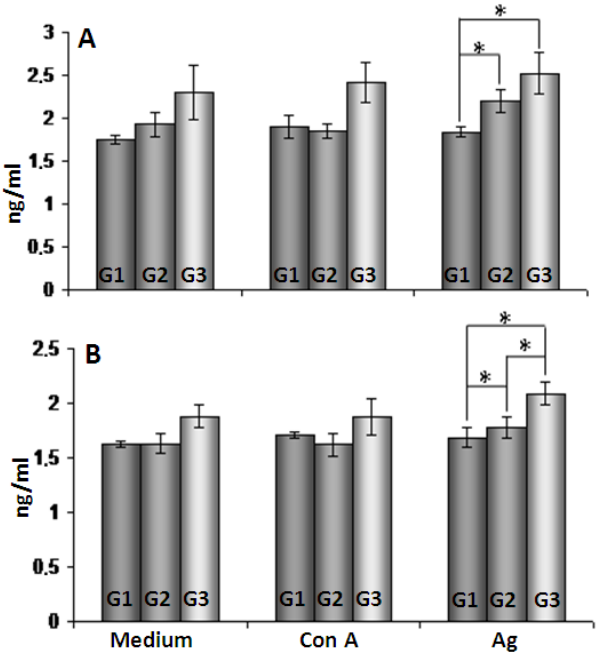

Fig. 3. Concentration of IL-2 produced *in vitro* by spleen (A) and mesenteric lymph node (B) cells derived from *A. ceylanicum* infected hamsters of different age: G1 – 4-week-old; G2 – 8-week-old; G3 - 6-month-old. Cells were cultured for 72 h in RPMI 1640 medium and stimulated with concanavalin A (ConA); *A.ceylanicum* somatic antigen (Ag) or unstimulated (medium). Statistically significant differences ($P<0.05$) are marked with an asterisk (*).

Discussion

In this study we report the cloning and sequencing of hamster IL-2 cDNA. The alignment of the coding sequence showed the highest homology (89.3%) to cotton rat sequence, other rodent species such as rat, deer mouse, and Mongolian jird sequences were also highly similar to the cloned hamster IL-2 sequence. The deduced amino acid sequence contained 155 aa. The signal sequence of 20 aa was identified as in all analysed IL-2 sequences. Two conserved cysteine residues forming a disulfide bond were found. This bonding stabilises the structure of the cytokine with N- and C-ends located close together, which is crucial for cytokine binding with its receptor (38). No N- or O-glycosylation sites were predicted as in human, Rhesus monkey, and deer mouse IL-2. Its analogs from other analysed species are glycosylated in at least one position. They all have a similar molecular weight of about 15 kDa except for mouse IL-2, which has a unique region consisting of 12 glutamine residues in a row at the N-terminal region (15). Alignment of amino acid sequences showed the highest similarity between hamster cotton rat and deer mouse IL-2. Another hamster cytokine, IL-12, which was cloned by Maruyama (27) also shows the highest homology to the cotton rat analog.

Expression of IL-2 was reported in many publications. Human IL-2 was produced in bacterial and eukaryotic cells (11, 35), mouse IL-2 was expressed in eukaryotic cells (15), bovine (8), duck (37), and goose (32) IL-2 in *E. coli* cells. In this study recombinant hamster IL-2 was produced in *E. coli* cells. The same method was previously used for expression of hamster IL-4, IL-12, and IFN-γ (12). However, in present experiment the purification of recombinant protein from bacterial cells by the method of affinity chromatography was not sufficient, as there were remaining bacterial proteins present in eluted fractions. Further purification by the method of electroelution from the polyacrylamide gel was applied with satisfying results, as the obtained rIL-2 sample was homogenous.

Immunisation of laboratory animals with recombinant cytokines is used with success to produce antibodies specific for cytokines of species, for which there are no commercial reagents. Morar *et al*. (30) cloned a rhinoceros IFN-γ, which was expressed in bacterial cells and the recombinant protein was used for production of polyclonal and monoclonal IFN-γ specific antibodies. In a similar way antibodies specific for dog IL-1β and TNF were produced (4).

Purified rIL-2 was used to immunise chickens in order to generate specific antibodies. Chickens were chosen for immunisations because this species is phylogenetically distinct from mammals, which is important when it comes to producing antibodies highly conserved between mammal species (21). Western blotting analysis showed that the produced antibodies recognised rIL-2. Moreover, antibodies recognised a single protein in splenocyte culture medium with a molecular mass of about 30 kDa, which is probably the dimeric form of IL-2. Since mouse IL-2 occurs rather as a dimeric form composed of two protein chains with

molecular weight of 16-18 kD (24) and human recombinant IL-2 also undergoes dimerisation, but only in high concentrations (1), it is possible that hamster IL-2 is also dimeric.

IL-2 production was investigated in *A. ceylanicum* infected hamsters. Spleen and mesenteric lymph node cells derived from younger hamsters released smaller amounts of cytokine than those isolated from adult 6-month-old animals. The same pattern was observed in our previous study concerning *in vitro* production of IL-4, IL-12, and IFN-γ by hamster splenocytes (12). This can be attributed to the immaturity of the immunological system in young hamsters or to the higher number of worms in their intestines, which suppress the immune response more efficiently. It has been proven that hookworm antigens efficiently inhibit proliferative responses of T cells in infected humans (16) and hamsters (29), which is coincident with lower production of IL-2 as this cytokine is responsible for T cell proliferation and differentiation. Hookworms also stimulate enhanced production of regulatory cytokines IL-10 and TGF-β (9, 17, 18, 29), which effectively down-modulate the immune response. Older and more resistant hamsters produce bigger amounts of IL-2, which probably results in higher proliferative responses of T cells and more effective immune responses against the parasite.

In conclusion, cloning, expression of recombinant protein and immunisation of laboratory animals is an effective method of IL-2 specific antibody production. Using these antibodies we have shown that young hamsters susceptible to *A. ceylanicum* infection produce less IL-2 than adult more resistant animals.

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