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Interleukin-2 (IL-2) expression in livers of patients with chronic hepatitis C virus (HCV) infection

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Abstract: The studies performed till now have pointed to an increased serum levels of interleukin 2 (IL-2) in infection with hepatitis C virus (HCV). The present study was aimed at examining intrahepatic expression of IL-2 in children (n=15) and in adults (n=11) with chronic hepatitis C as well as its correlations with histological lesions and selected clinical data. The immunocytochemical techniques and *in situ* hybridization method were applied at light and electron microscopy level. Under the light microscope, expression of IL-2 was analysed semiquantitatively. As compared to the control material, in livers of both groups of chronic hepatitis C patients augmented expression of IL-2 was demonstrated. The reaction product was localized mainly in the cytoplasm of hepatocytes which was confirmed by hybridocytochemistry. The mean proportion of cells with positive reaction for IL-2 mRNA was significantly lower than the proportion of cells positive for the respective protein. No correlation was disclosed between IL-2 expression on one hand and grading or staging, alanine aminotransferase (ALT) and HCV RNA levels in serum on the other. At the ultrastructural level, IL-2 in hepatocytes was present mainly in the endoplasmic reticulum and mitochondria. Our studies have confirmed augmented expression of IL-2 in livers of patients with chronic hepatitis C and have demonstrated that hepatocytes represent the principal source of the cytokine in HCV *in vivo* infection. Moreover, expression of IL-2 in the infection was examined for the first time at the ultrastructural level. Mitochondrial localization of IL-2 suggests a direct involvement of the cytokine in disturbed function of the organelles. (www.cm-uj.krakow.pl/FHC)

Key words: Interleukin 2 - Chronic hepatitis C - Immunocytochemistry - Immunoelectron microscopy - *In situ* hybridization

Introduction

Ineffective immune response is suggested to play a principal role in the pathogenesis of chronic HCV infection. It has been proposed that the immune response in chronic hepatitis C is compartmentalized, with a predominantly Th2 or Th0 response at the periphery and a Th1 response in the liver [3, 5, 19, 21]. The Th1 cytokines include interleukin 2 (IL-2), the cytokine most important for cell growth and survival of T-lymphocytes, natural killer (NK) cells and some non-lymphoid cells but also known to sensitise T cells to apoptosis [18]. A significant number of patients with chronic hepatitis C had elevated IL-2 levels [4]. In chronic hepatitis C, IL-2 expression was noted to correlate with grading and staging [7, 21]. Higher expression of mRNA for the cytokine was observed also in patients who failed to react to interferon α (IFN-α) treatment [7], but circulating levels of IL-2 did not predict the response to IFN-α treatment [4]. However, reports are also available on unsuccessful search for genomic IL-2 expression in liver with HCV infection [20]. HCV itself may directly affect genomic control of IL-2 and of other cytokines and in this way disturb immune processes in the liver [2, 32]. The role of hepatocyte-derived cytokines remains unknown. Our earlier hybridocytochemical studies on mRNA for cytokines in HCV infection demonstrated transcripts for TNF-α and IL-1α mainly in cells of liver sinusoids and of inflammatory infiltrates, but weaker signal was also demonstrated in hepatocytes [13].

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The aim of the present study was to investigate the intrahepatic expression of IL-2 and its mRNA in chronic hepatitis C in children and adult patients. Our study was also aimed at the analysis of subcellular localization of IL-2 in liver biopsy material, since no data on the subject are available in the literature. Attempts were also made to correlate the intensity of cytokine expression with (a) inflammation activity (grading) and/or advancement of fibrosis (staging), (b) serum alanine aminotransferase (ALT) levels in groups of adult patients and children and (c) serum HCV RNA levels in children.

Materials and methods

Human liver specimens. The material included liver biopsies obtained from children (n=15) and adults (n=11) with chronic hepatitis C and with serologically confirmed active replication of the virus. Anti-HCV tests using ELISA third generation assay (Abbott Park, Illinois, USA) were positive in all children and all adults, HCV RNA was found in all children (subject to quantitative appraisal in 13/15 patients) and in all adults. In two children and all adults, qualitative assay only for HCV RNA was performed using Amplicor HCV test, version 2.0 (Roche, Mannheim, Germany). The test was standardised according to the WHO International Standard for HCV RNA. Quantitation of HCV RNA was conducted using Digene Sharp Signal System (Gacthersburg, MD 2878 USA 1201, Cloppe Road). The mean age of children (9 males, 6 females) at the time of liver biopsy was 14±1 year (range, 7-17 years) and the mean age of adult patients (3 males, 8 females) was 44±5 years (range, 22-72 years). All the tests were routinely performed for diagnostic purposes and before the anti-viral therapy**.** The biopsies originated from children of the Outpatient Clinic of Liver Disease, Specialized Unit for Mother and Child Health Care in Poznan, Poland and from adult patients of the Chair of Gastroenterology and Human Nutrition, University of Medical Sciences in Poznan and Ward of Infectious Diseases, Józef Strus Hospital in Poznan, where the biopsies were performed in 1999-2003. All patients were seronegative for both HBsAg and HBeAg antibody, and for IgM antibody against cytomegalovirus (CMV) or against Epstein-Barr virus (EBV) in standard enzyme immunoassays (ELISA). Other cases of liver damage (*e.g.* α_1-antitrypsin deficiency, Wilson's disease, alcohol dependency) were ruled out. All the patients exhibited elevated serum alanine aminotransferase (ALT) level (normal level below 40 IU/L) for at least 6 months. The control group consisted of liver biopsies of serologically HCV-, HBV-, HCMV-, EBV- and HIV-negative patients with non-specific changes in the liver (n=4 , children) or chronic hepatitis of unknown etiology (n=4, adults) (mean age of the control group was 24±8 years; range 9-53 years). All the control patients demonstrated normal serum ALT activity (below 40 IU/L). Paraffin sections of reactive lymph nodes from ten patients without liver disease were used as a positive control.

Tissue preparation. In total, 26 liver biopsy specimens were obtained during routine diagnostic procedures after receiving informed consent of the patients. For light microscopy, tissue specimens were fixed in 10% formalin and embedded in paraffin. Histopathological lesions were evaluated following the classical H+E staining as well as silver technique and trichrome technique according to Masson and periodic acid-Schiff with diastase pretreatment. At least 10 sections were prepared from each biopsy. Each biopsy specimen was evaluated using a simple numerical scoring system for the grade of portal/periportal necroinflammation (G1) (0-4), the grade of lobular necroinflammation (G2) (0-4), and for the stage of fibrosis (S) (0-4), as proposed by Scheuer *et al.* [26]. The histological lesions considered to be characteristic of chronic hepatitis were also evaluated. Histological evaluation was performed independently by two experienced histopathologists.

For electron microscopy, the material was fixed in paraformaldehyde/glutaraldehyde, embedded in epon and ultrathin sections were subjected to a conventional electron microscopy and immunoelectron microscopy [27].

***In situ* hybridization.** For *in situ* hybridization studies, 5 μm thick sections were cut and mounted onto SuperFrost/Plus microscope slides. The digoxygenin-labelled oligoprobe (oligo.pl, IBB PAN, Warszawa, Poland) specific for human cytokine IL-2 mRNA, showing the following sequence: 5'-Dig-TGG TGA GTT TGG GAT TCT TG-3' was used in dilution 1000 ng/ml and detected with sheep anti-digoxygenin monoclonal antibodies (MAbs) (Fab fragments), labelled with horseradish peroxidase (Roche, Mannheim, Germany). In the hybridocytochemical studies, both classical and amplified by the ImmunoMax technique [12, 15], sequential sections of the tissue material pre-tested by immunocytochemical techniques were used. The *in situ* hybridization protocol of R&D Systems was employed, with our own modification and ImmunoMax amplification of the signal. The subsequent stages involved paraffin removal from the section using xylene, ethanols and wash in diethyl pyrocarbonate (DEPC) (Sigma Chemical, St. Louis, USA)-treated distilled water, each lasting 1-5 min, followed by washing in 0.05 M TRIS (AccuGene, Maryland, USA) for 5 min at room temperature (RT). Endogenous peroxidase was blocked by 15 min incubation of the section in 1% H_20_2 at RT. Then, the sections were digested with proteinase K (DAKO, Carpinteria, CA, USA), 100-200 (μg/ml for 10-15 min at 37°C. Subsequently, the sections were washed in PBS for 10 min at RT, fixed again in 0.4% PFA at 4°C for 15 min and washed in DEPC-distilled water for 1 min. The prehybridization solution (Sigma) was applied under a coverslip and the preparation was incubated for 1 h at 37°C. Various concentrations of the oligoprobe were tested, ranging from 100 to 1000 ng/ml, dissolved in the prehybridization solution plus 30% formamide. The proper hybridization took place overnight (16 to 18h) at 38°C, under a coverslip, in a humid chamber. After removing of the coverslips, the sections were washed in decreasing concentrations of SSC (Promega Corp., Madison, WI, USA) plus 30% formamide (Sigma) and, then, in 0 .1M Tris-HCl, pH 7.6 and 0.1% Triton X-100 in TBS for 15 min at room temperature. Sheep anti-digoxygenin MAb-horseradish peroxidase (HRP) (Roche) complex was applied for 45 min at RT in dilution 1:30. After a wash in PBS/Tween20, biotinylated tyramine (PerkinElmer Life Sciences, Inc., Boston, MA, USA) was applied at 1:50 dilution for 3 min at RT. The streptavidin-peroxidase complex was added for 30 min at RT. The hybrids were detected using peroxidase substrate, 0.05% DAB (DAKO) in 0.05 M Tris/HCl, pH 7.6 supplemented with 0.001% H_20_2, for 5 min at RT. Some sections were counterstained with hematoxylin.

As controls for *in situ* hybridization, lymph nodes from ten donors without liver diseases served as initial positive controls. Negative controls included (a) hybridization without addition of the molecular probe and (b) incubations of slides in an RNase A solution (R&D Systems), 20 mg/ml in 2xSSC/10 mM $MgCl_2$, pH 8, for 30 min at RT before hybridization. All the experiments were performed at least twice.

Immunocytochemistry. For immunocytochemistry, the following specific MAbs were used: (a) mouse anti-human IL-2 antibody (1:100) (R&D Systems), (b) mouse anti-human CD68 and anti-human Von Willebrand factor (DAKO EPOS A/S, Denmark) (both ready for use dilutions) (DAKO, Glostrup, Denmark). The studies followed the classical avidin-peroxidase complex (ABC) technique [9], alone or associated with the ImmunoMax technique [15], described in detail in our earlier papers [13]. As controls for immunocytochemistry, lymph nodes from ten donors without liver diseases served as initial positive controls. Most of the sections were subjected to double immunocytochemical reactions, including macrophage

marker (CD68) or marker of endothelial cells (Von Willebrand factor) and IL-2. The terminal reaction product was visualised using DAB (brownish-black signal) or DAB supplemented with nickel (blue colour). Some sections were counterstained with hematoxylin. Results of the IL-2 expression and expression of its transcript in reactive lymph nodes (positive control) were not subjected to semiquantitative analysis but allowed for quality appraisal of IL-2-specific antibodies and oligoprobes, used in principal studies.

Immunoelectron microscopy. At the electron microscope level, the immunocytochemical tests were preceded by examination of IL-2 protein expression on semithin sections using ABC and ImmunoMax techniques. Ultrathin sections were subjected to a labeling with 15 nm colloidal gold-streptavidin (Biocell Int., Cardiff, UK). The negative control of the immunocytochemical reaction at the level of electron microscopy involved substitution of primary antibodies by a normal serum obtained from the same animal as the primary. Moreover, the company which produced the antibodies (R&D Systems) warranted specificity of the antibodies (ELISA tests, results of Western blot analysis).

Semiquantitative and statistical analyses. The contents of IL-2 and of respective mRNA in liver biopsies demonstrated using ABC method and hybridocytochemistry, both combined with ImmunoMax technique, were assessed by a semiquantitative technique relating the score of 0 to 4 points to the fraction of stained cells (score 0 - 0% cells, 1 - less than 5% cells, 2 - 5-20% cells, 3 - 20-40% and 4 - more than 40% positive cells). It should be stressed that the semiquantitative analysis was performed exclusively on sections stained using the ImmunoMax technique (both in the case of immunocytochemistry and *in situ* hybridization), in which intense colour reaction was observed. The slides were examined under a light microscope, at × 400 magnification. In every section of the liver biopsy, five fields in hepatic lobules and periportal area and at least five different portal spaces in each specimen were examined. The calculations were performed on the mean of three consecutive sections of liver biopsy material from HCV-infected patients and resulted in mean values, separately for lobular/periportal area and for portal area. Cellular localization of the studied proteins and the respective mRNA was evaluated (cell nucleus, cytoplasm, membranes). StatXact Software (Cytel Software Corp.) was used for statistical analysis of small data sets. In order to determine statistical significance of variations in the percentage of cells positive for mRNA-IL-2 and proteins studied, we first calculated the mean values of immunostaining or hybridocytochemical signal scores for both liver biopsy groups (adults and children) as well as for control biopsy specimens, separately for lobular/periportal area and portal spaces. The values were then compared using the Mann-Whitney U-test for nonparametric independent data and the Wilcoxon test for nonparametric dependent data. Correlations between data rows were determined employing Spearman's rank correlation index. In determining the correlations, means of the total expression of cytokine mRNA or of the respective protein were taken into account (*i.e.* expression in the lobules, periportal and portal spaces) and related to the sum of grading (G1+G2), staging (S) and ALT, HCV RNA, respectively, in the groups of children and adult patients.

Results

Histology of liver biopsy material

All 26 liver biopsy samples of anti-HCV and HCV-RNA positive patients were diagnosed by histological criteria as consistent with chronic hepatitis C. Both the grade of inflammation and the stage of fibrosis were significantly lower in children than in adults (grade of inflammation (G1+G2), 1.8±0.3 vs. 3.4±0.3, respectively, $p<0.003$) and stage of fibrosis, 1.0±0.2 vs. 2.6±0.3, respectively, $p<0.0002$). In histological patterns, large groups of lymphoid cells in dilated portal spaces prevailed in adult patients (10/11). The infiltrates consisted of individual lymphoid cells or of their groups. In control liver biopsies, nonspecific changes were noted, with individual cells of inflammatory infiltrate in portal spaces (final grading, 1.3±0.2), with no traits of liver fibrosis (n=6) or with fibrosis restricted to portal spaces (n=2) (mean staging for all patients was 0.3±0.1).

Table 1. Score of histological diagnosis, selected clinical data and semiquantitative assessment of IL-2 and IL-2 mRNA in liver biopsies of children with chronic hepatitis type C

Patient, age (yrs), sex	Score of histological diagnosis[1]			IL-2[2,3]		ALT (U/L)	HCV RNA (pMol)
	G1	G2	S	mRNA	protein		
1. 14,M	1	0	0	1/1	4/0	56	0.759
2. 7,F	1	2	3	1/1	4/0	177	0.782
3. 15,F	1	0	2	1/0	4/0	185	nt
4. 15,F	1	0	0	1/0	3/0	206	0.733
5. 15,M	1	2	0	2/0	4/0	53	0.873
6. 13,M	1	1	1	1/1	3/0	60	nt
7. 15,F	1	0	1	2/1	4/1	96	0.690
8. 16,M	2	1	1	2/1	4/1	88	0.705
9. 14,M	1	1	1	1/1	4/0	37	0.753
10. 17,M	1	0	1	1/0	3/0	68	0.423
11. 17,M	0	0	1	1/1	3/0	73	0.496
12. 15,M	2	1	1	1/1	1/1	95	0.383
13. 12,M	2	1	1	2/1	4/0	68	0.373
14. 8,F	2	0	1	2/1	4/0	18	0.446
15. 14,F	1	0	1	2/0	3/0	24	0.159

Designations: G1 - grading in portal spaces; G2 -grading in lobules; S- staging; [1]Scoring system: see Material and Methods; [2]Score: 0 = negative signal; 1 = less than 5% positive cells; 2 = 5-20% positive cells; 3 = 20-40% positive cells; 4 = more than 40% positive cells; nt - not tested; [3]The results are expressed by two numbers of which the first denotes expression in lobules (Kupffer cells, endothelium, hepatocytes) and the other - expression in inflammatory infiltrates in portal spaces (lymphocytes, macrophages, endothelium)

In situ hybridization

mRNA for IL-2 was demonstrated in all children and in 6/7 examined adult patients (Table 1, 2). Transcript for IL-2 was located in cells of liver sinusoids (macrophages, endothelial cells), in some cells of inflammatory infiltrates (lymphoid cells, macrophages) and in some hepatocytes (Fig. 1A,B). The reaction product was de-

Table 2. Score of histological diagnosis, selected clinical data and semiquantitative assessment of IL-2 and its mRNA in liver biopsies of adults with chronic hepatitis type C

Patient, age (yrs), sex	Score of histological diagnosis[1]			IL-2[2,3]		ALT (U/L)
	G1	G2	S	mRNA	protein	
1. 41,F	2	2	2	1/0	3/1	53
2. 22,M	1	2	2	2/0	2/0	88
3. 39,M	1	2	2	1/0	3/0	212
4. 55,F	3	2	4	2/1	4/0	100
5. 51,F	2	3	4	1/0	4/0	85
6. 54,F	2	1	3	nt	3/2	91
7. 56,F	2	2	2	nt	4/0	56
8. 72,F	2	2	2	nt	4/0	72
9. 43,F	1	1	1	nt	4/1	43
10. 27,F	1	1	4	0/0	3/1	100
11. 24,M	1	1	1	1/0	4/1	40

Designations: G1 - grading in portal spaces; G2 -grading in lobules; S- staging; [1]Scoring system: see Material and Methods; [2]Score: 0 = negative signal; 1 = less than 5% positive cells; 2 = 5-20% positive cells; 3 = 20-40% positive cells; 4 = more than 40% positive cells; nt - not tested; [3]The results are expressed by two numbers of which the first denotes expression in lobules (Kupffer cells, endothelium, hepatocytes) and the other - expression in inflammatory infiltrates in portal spaces (lymphocytes, macrophages, endothelium)

Table 3. Comparative hybridocytochemical and immunocytochemical scoring of IL-2 and its mRNA in children vs adults with chronic hepatitis C and in HCV-infected patients vs control biopsy specimens

Cytokine	L+PS		
	children (n=15)	adults (n=11)	control (n=8)
mRNA-IL-2	2.07 ± 0.18^{A}	1.29 ± 0.29^{B}	0.25±0.14
IL-2	$3.60\pm0.21^{*,A}$	$4.00\pm0.27^{**,C}$	0.38±0.16

Data are means ±SE Designations: L+PS - cytokine expression in hepatic lobules and in portal spaces; *- p<0.002; **- p<0.001 expression of mRNA vs that of respective protein in each group of patients; A - p<0.001- expression in the children vs that in the control; B - p<0.03 and C - p<0.001 - expression in adults vs that in the control

Table 4. Comparative hybridocytochemical and immunocytochemical scoring of IL-2 and its mRNA in hepatic lobules (L) and portal spaces (PS) in children vs adults with chronic hepatitis C

Cytokine	children (n=15)		adults (n=11)	
	L	PS	L	PS
mRNA-IL-2	1.40±0.13	0.67±0.13	1.14±0.21	0.14±0.11
	p<0.01		p<0.03	
IL-2	3.40±0.21	0.20±0.11	3.45±0.21	0.55±0.218
	p<0.001		p<0.004	

Data are means ±SE

tected in the cell cytoplasm. A significantly higher total expression of mRNA for IL-2 was noted in biopsies from children and adults with HCV infection as compared to control liver biopsies (p<0.001; p<0.003, respectively). As compared to the IL-2 protein-immunopositive cells, much lower percentage of the cells with the transcript was seen in children (p<0.002) and in adult patients (p<0.001) both in the lobules and in the portal spaces (Table 3). No significant differences between children and adults were detected in the percentage of cells with total transcript for the IL-2 (Table 3). In control liver biopsies, mRNA for IL-2 was seen in 2/8 patients.

Comparative analysis demonstrated a significantly higher amount of IL-2 mRNA in lobular localization as compared to portal localization both in children and in adults with chronic hepatitis C (p<0.01; p<0.03, respectively) (Table 4).

Immunocytochemistry

IL-2 was detected in all studied HCV-positive patients. No significant differences were disclosed in IL-2 expression between children and adults even if the latters showed much more pronounced grading and staging. The immunoreactivity for IL-2 was observed mostly in hepatocytes and only in individual sinusoidal cells or inflammatory cells. The signal was strong, either evenly distributed in cytoplasm of hepatocytes or localized in one of the cell poles (Fig. 2A). The source of IL-2 and of its mRNA was identified by double staining. As a rule, immunocytochemical product for IL-2 protein was localized in hepatocytes and very rarely in cells of liver sinusoids (Fig. 2B). In the control liver biopsies, only scattered individual IL-2-positive cells were detected among hepatocytes and sinusoidal cells.

In the positive control material (reactive lymph nodes) co-expression of mRNA for IL-2 and of the protein itself was demonstrated in a variable number of lymphocytes, depending on the tested lymph node (Fig. 3).

The studies confirmed higher sensitivity of the ImmunoMax technique, as compared to classical immunocytochemical and hybridocytochemical techniques.

Immunoelectron microscopy

Application of ABC technique and ImmunoMax technique on semithin sections confirmed expression and cellular localization of IL-2 (Fig. 4). At the ultrastructural level, application of colloidal gold permitted to document the presence of the protein mainly in enlarged mitochondria, dilated ER cisternae and the hepatocyte cytoplasm (Fig. 5A,B).

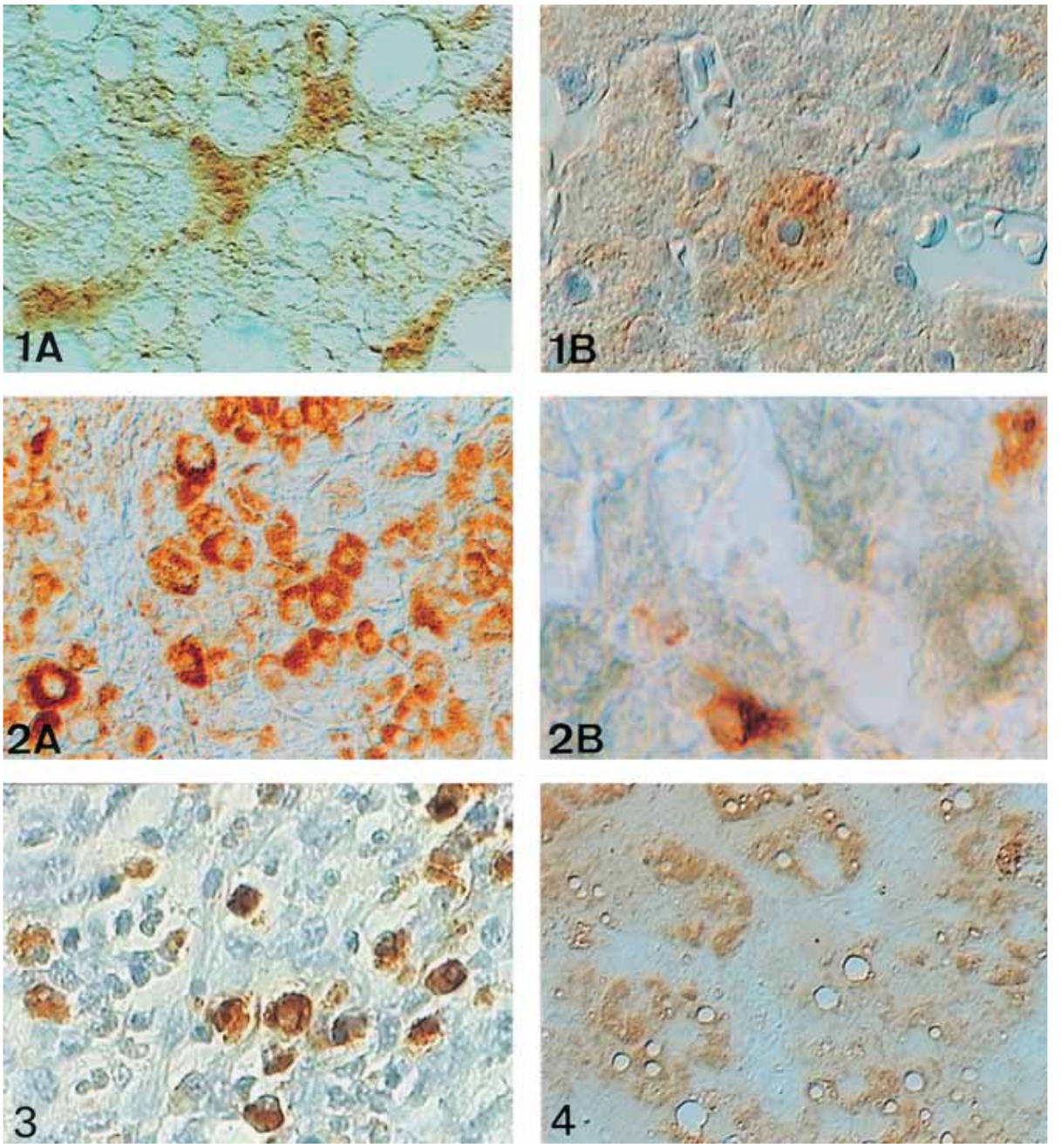

Fig. 1. Fragments of hepatic lobules from patients with chronic HCV infection (A) mRNA for IL-2 in liver sinusoidal cells and (B) in individual hepatocytes. *In situ* hybridization, ImmunoMax technique. × 400. **Fig. 2.** Fragments of hepatic lobules from patients with chronic HCV infection (A) localization of IL-2 in many hepatocytes (B) double staining for IL-2 expression (blue-grey hepatocytes) and with anti-CD68 antibodies (brown macrophages). A: × 400, B: × 1000. **Fig. 3.** Localization of IL-2 in immunoreactive lymph node (positive control material). ABC-ImmunoMax technique. × 400. **Fig. 4.** IL-2 localization on semithin section in hepatocytes. ABC-ImmunoMax technique. × 400.

Correlation of IL-2 expression and grading/staging, ALT or HCV-RNA

In none of the patient groups could significant correlation be disclosed between IL-2 expression (protein and transcript) on one hand and grading/staging, ALT or HCV RNA on the other. In children, the correlation between total IL-2 expression and serum HCV RNA level was the closest to approach the level of significance. No attempts to examine such a correlation could

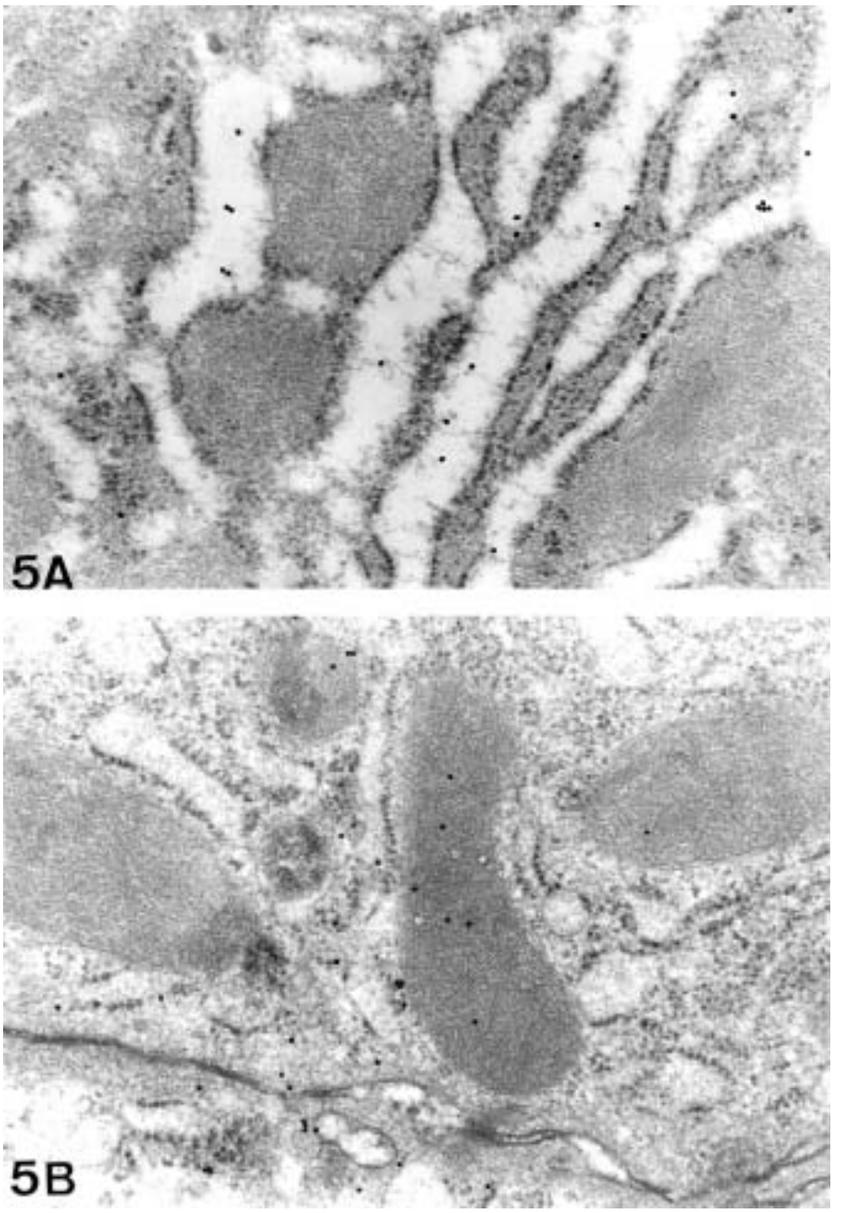

Fig. 5. Ultrastructural localization of IL-2 protein in hepatocytes. (A) Gold particles overlay mainly ER cisternae. (B) Gold particles overlay mainly dilated ER cisternae and enlarged mitochondria. Streptavidin-colloidal gold. TEM, A: × 30 000; B: × 25 000.

be made in adult patients, since in the latters only the qualitative serum HCV RNA assays were available. In the HCV-infected adults, the correlation between total IL-2 expression and ALT activity was the closest to reach the level of significance.

Discussion

HCV continues to represent the main causative agent of the hepatitis which - independently of patient's age - leads to chronic transformation of the process in over 80% patients [1]. In the patients an increase in serum levels of pro-inflammatory cytokines [17, 30] and up-regulation of intrahepatic Th1-like cytokines [21] have been noted. Hepatitis C virus may be resistant to inhibition by cytokines, so they can play a more prominent role in liver damage than in controlling or terminating the viral replication [16, 28]. The *in vitro* studies demonstrated a variable effect of HCV proteins on expression of IL-2 gene [2, 32].

The present study represents a continuation of investigations on IL-2 expression in chronic hepatitis C, dealing in particular with detection of mRNA for IL-2 and with the localization of the cytokine at ultrastructural level. IL-2 is known to be produced mainly by Th1 cells and its role involves stimulation of proliferation and activation of most T lymphocytes, NK cells and B lymphocytes. IL-2 is also known to function in some non-

lymphoid cells [24, 29]. The normal and neoplastically transformed epithelial cells are also capable of expressing endogenous IL-2, processed and secreted in the same way as in hematopoietic cells [25]. Contradictory opinions have been expressed on the amounts of cytokines detectable in livers of patients with chronic hepatitis C and on correlation of the cytokine expression on one hand with grading and/or staging or intrahepatic viral load on the other [5, 8, 17, 21]. No comparative data are available on the intrahepatic cytokine expression in chronic hepatitis C in children and in adults. Our earlier investigations have shown more pronounced expression of IL-1α, TNF-α and IL-2 in chronic hepatitis C, and the proteins have been detected mainly in hepatocytes [13]. The augmented IL-2 expression in patients with chronic hepatitis C used to be taken as a proof for intrahepatic Th1 response but cellular sources of the cytokine have not always been examined [5, 19, 21]. The very low expression of cytokines in cells of inflammatory infiltrates in our patients (both in portal spaces and in the lobules) might have reflected weak stimulation of mononuclear cells, low number of infiltrating activated macrophages (CD68) or disturbed production of pro-inflammatory cytokines by cells of the infiltrate. Studies performed *in vitro* using the protein NS3 to stimulate responses of T cells from infected individuals have shown both, the absence of IL-2 protein secretion by the lymphocytes and the failure to amplify IL-2 mRNA upon stimulation [6]. Subsequently, various immunodominant epitopes of NS3 protein have been demonstrated, which favour IL-2/interferon-γ or IL-10 secretion, respectively [32]. Other authors indicate that the full-length but not truncated C protein of HCV activates transcription from the IL-2 promoter in T cells [2]. Results of the studies may explain the differences in correlations observed *in vivo* or in absences of such correlations between increased IL-2 expression and increased IFN-γ expression, grading and staging in chronic hepatitis C infection [21].

In the present study, the ImmunoMax technique has been applied for the first time in investigations on IL-2 expression in the liver. It has amplified sensitivity of the classical imunocytochemical techniques and of *in situ* hybridization. We have documented augmented expression of IL-2 in patients with chronic hepatitis C as compared to the control liver biopsies. Furthermore, IL-2 expression was compared between children and adults with chronic hepatitis C but no significant differences could be detected even if histologically detectable lesions were much more pronounced in the adult patients. We have confirmed our earlier observations on IL-2 expression in hepatocytes, extended at present by ultrastructural studies which have demonstrated localization of IL-2 in regions of endoplasmic reticulum and in mitochondria . Hepatocytes proved also to be the source of other cytokines in HCV infection, including IL-1α, TNF-α and IL-6 [8, 13, 23]. At the ultrastructural level, only localization of IL-6 has been documented till now, mainly in endoplasmic reticulum cisternae [11, 23]. Mitochondrial localization of IL-2, observed in the present investigations, points to a potential role of the cytokine in damage induced to the organelles and in morphological lesions, frequent in ultrastructural descriptions of the infection [10]. Semiquantitatively detected higher expression of IL-2 in HCV infections, as compared to the non-HCV infections in the liver, and the presence of the expression also in mitochondria in chronic C hepatitis, may suggest its direct or indirect relationship with compromised function of the organelle, *e.g.*, in the scope of fatty acid metabolism, for which, *i.a.*, cytochrome P450 is responsible. *In vitro* studies proved that down-regulation of cytochrome P450 reflects also over-expression of c-myc in rat hepatocytes, resulting from administration of exogenous IL-2 [31]. The disturbed structure of mitochondria has already been described in our earlier studies on ultrastructural patterns noted in chronic HCV infection [10]. The mechanism (primary or secondary) of the mitochondrial damage remains unknown. Our team has also described localization of some HCV proteins (NS3, C) within mitochondria [14]. The damaged function of mitochondria is frequently linked to augmented cell levels of oxygen free radicals (ROS) and secondary lipid peroxidation and may show links to the frequently observed phenomenon of lipid degeneration of hepatocytes in this viral infection [22].

Augmented expression of IL-2 in hepatocytes of patients with chronic hepatitis C, as compared to control livers, points to a significant involvement of the cytokine in pathogenesis of chronic hepatitis C, independently of the advancement of histological lesions (grading/staging).

The significantly lower expression of mRNA for IL-2, as compared to expression of the respective protein, may reflect lower actual production of the transcript and/or its higher sensitivity to degradation in paraffin sections.

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