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IMPAIRMENT OF RECENT THYMIC EMIGRANTS IN HCV INFECTION

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Hepatitis C Virus (HCV) often has a more favorable course in younger patients. Considering the involution of the thymic function with age, we investigated the output of recent thymic emigrants (RTE) in HCV patients. To evaluate RTE, we used a competitive quantitative PCR in order to determine the percentages of cells with cj-T cell receptor excision circles (TREC). This study was performed in 14 HCV patients at diagnosis and before any anti-HCV treatment. The results obtained in this group were compared to those obtained in a group of age-matched controls. We found that in the 14 HCV patients naive for anti-HCV treatment the mean percentage of cj-TREC was 3%. We could not detect a correlation between the percentages of cj-TREC and age or patients' viremia. In contrast, in the 26 age-matched controls mean percentage of cj-TREC was 5.6% (P=0.01). Our study describes a novel immune defect in HCV patients. Additional studies are needed to get further insight in the possible role of TREC defect in the pathogenesis and prognosis of the disease.

Hepatitis C virus infection (HCV) is a major issue in world public health, affecting approximately 2% of the world's population (1). The infection has a variable clinical course. The outcome has been related to the evolution of the viral quasispecies (2). Since HCV replicates in lymphatic tissue, several immunological disorders, linked to persistent antigenic stimulation, are associated to patients with chronic active hepatitis. Apart from the viral factors, the immune mechanisms responsible for the highly variable natural history in a given patient are only partially known. Some reports have pointed out a more favorable prognosis in younger patients (3-4). T-lymphocytes are of pivotal importance in host defense against viruses. T-cells are produced in the thymus and this organ undergoes a physiological involution with age.

In the last decades, it became possible to evaluate the thymic output, measuring the T-cell receptor excision circles (TREC) present in the so called "recent thymic emigrants" (RTE) (5-6). TREC are DNA fragments representing a byproduct of T-cell receptor rearrangement; there are at least two possible molecules, named coding-joint (cj) and signal-joint (sj) TREC, each of which is produced in a defined moments of the intrathymic maturation of T cells. Since their DNA exists in a circular form, they are not duplicate during mitosis, but progressively diluted in peripheral blood T-cells. TREC are present only in virgin cells, i.e. those newly produced in the thymus, and thus their measurement allows the determination of RTE. These cells have been proved to be altered in several clinical conditions, with clinical and prognostic implications (7-9).

For these reasons, we aimed to verify the hypothesis of thymic impairment in patients with HCV infection by analysis of means of RTE in a prospective case-control study.

Key words: Hepatitis C Virus (HCV); recent thymic emigrants (RTE); cj-T cell receptor excision circles (TREC).

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PATIENTS AND METHODS

Patients

To avoid possible confounding factor in the analysis of the thymic function, the following exclusion criteria were used: 1. thymoma; 2. presence of autoantibodies positivity; 3. coexistence of other infectious diseases; 4. autoimmune liver disease, alfa1-antitrypsine deficiency, Wilson's Disease; 5. drugs and/or toxic liver disease; 6. alcohol assumption > 20 g/day; 7. presence of cryoglobulins; 8. coexistence of other autoimmune diseases or lymphatic disorders; 9. diabetes; 10. celiac disease, as assessed by the positivity of anti-antiendomisium or tissue transglutaminases IgA; 11. first degree relatives with clinical history of immunological disorders; 12. assumption of immunomodulating drugs in the last year; 13. clinical evidence of cirrhosis.

According to the above criteria, we enrolled 14 consecutive non-smoker Caucasian patients coming to our Outpatient Liver Unit for their first diagnosis of hypertransaminasaemia and serological positivity for HCV infection without previous history of antiviral treatment and fitting the above criteria for the study. After informed consent, as authorized by local Ethical Committee in accordance with the Helsinki Declaration of 1975, a complete medical history was obtained and a physical examination performed by a single physician. On the day of liver biopsy for the staging of chronic hepatitis, all patients underwent laboratory evaluation of biochemical and liver function tests. A blood sample was withdrawn from the patients for RTE assessment.

During the same period, a group of 26 healthy volunteers (mean age: 49.0; range 26-69), matched for age and sex, with normal liver function tests and negative serology for HCV, from medical staff of the same hospital served as controls. Exclusion criteria for the enrollment were the same as the HCV+ patients.

RTE determination

PBMC were isolated from heparinized venous blood, according to standard procedures. Genomic DNA was extracted from PBMC with a commercially available kit (Qiagen, Hilden, Germany) according to manufacture's recommendations. The number of copies of cj-TREC was measured as previously reported (10) by a quantitative-competitive PCR. In brief, for each sample, a separate PCR reaction was performed by using primers specific for FasL in order to estimate the total amount of genomic DNA. Each reaction mixture contained 10x PCR buffer (Promega, Madison Wisconsin, USA), dNTP 2mM, primers cj-DIR and cj-REV 200mM, $MgCl_2$ 1.5 mM, Taq Polymerase 1U (Promega, Madison, Wisconsin, USA). The final volume of the reaction was 25 microliters. To each PCR reaction cj-TREC/ng DNA internal standard at various concentration were added (internal standard had the same prime–binding as the template but was genetically modified to be 50bp shorter for cj-TREC and 36 bp shorter for FasL).

PCR was performed in Thermal Cycler (BIO-RAD, Hercules, California, USA) by using the following conditions: 1 cycle of denaturation (95°C for 5 minutes) followed by 45 cycles of amplification (30s at 94°C, 30s at 50°C, 42s at 72°C) and a final 7 minutes extension at 72°C. The PCR product was loaded on 3% agarose gel and run at 90 V for 60', stained with ethidium bromide and analysed by a Gel Doc 2000 (BIO-RAD, Hercules, California, USA) video densitomer. The competitor band was observed as a lower 310 bp band in respect to the 360 band of the wild type, as shown in figure 1. The relative intensity of competitor band was corrected by 1.16 factor to compensate minor ethidium bromide incorporation. The ratio between the lower and upper bands was linearly correlated with the input number of competitor molecules and number of copies of wild type DNA was calculated with the same technique, using the same competitor at identical concentrations and the same PCR mix; however in this case primers 276D and 60R that detect CD178 were added to the mix. The percentage of cj-TREC positive cells was finally calculated as the ratio between the number of copies of nuclear DNA multiplied by 2, because two copies are present in the genome.

Statistical Analysis

The nonparametric *Wilcoxon* signed rank was used to compare healthy controls with HCV patients. Analysis was performed with *Statview 4.5* software for the Macintosh.

RESULTS

Relevant clinical and laboratory data of the patients are summarized in Table 1. Viremia was available in 10 patients (mean: 1.411.000; range: 5100-3.334.000 UI/ml). Percentages of cj-TREC-positive cells in relation with age are shown in Figure 2. The distribution of cj-TREC percentages (range 0-12%) showed considerable variations, but correlation between percentages of cj-TREC-positive lymphocytes and age of the patients was not observed. No correlation between cj-TREC percentage and viremia was observed (not shown). On the other hand, a significant negative correlation between cj-TREC percentage and age was present as expected in the control group (P=0.02). cj-TREC percentages in HCV patients and controls are shown

Table I. *Relevant clinical and laboratory data of the study population*

	HCV + patients (*n=14*)	**Healthy controls (*n=26*)**	***p***
Age (mean ± S.E.*)	48.3 ± 3.7	49.0 ± 3.2	n.s.
Sex (M/F)	7/7	12/14	
Anti-HCV +ve	14 (100%)	0	
HCV Genotype			
- 1b	11 (78,57%)	-	
- 3a	3 (21,43%)	-	
- Others	0	-	
ALT (nv:45UI/L)	115.52 ± 94.75	25.47±12.33	0.001
AST (nv:45UI/L)	75.41 ± 153.75	19.17±8.345	0.001
GGT (nv:35UI/L)	71.85 ± 45.54	28.49±9.17	0.001

**S.E. Standard error*

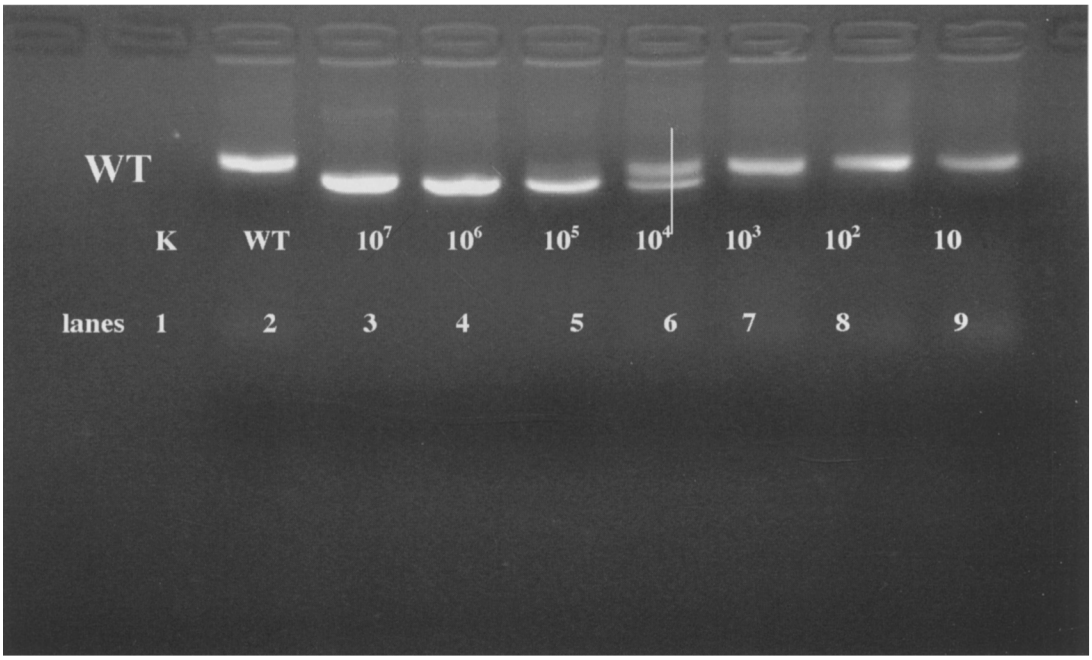


Fig. 1. *PCR product of cj-TREC amplification. Lane 1, control (K); lane 2, wild type (WT); lanes 3-9, competitor at various concentrations. The competitor band was observed as a lower 310 bp band in respect to the 360 band of the wild type.*

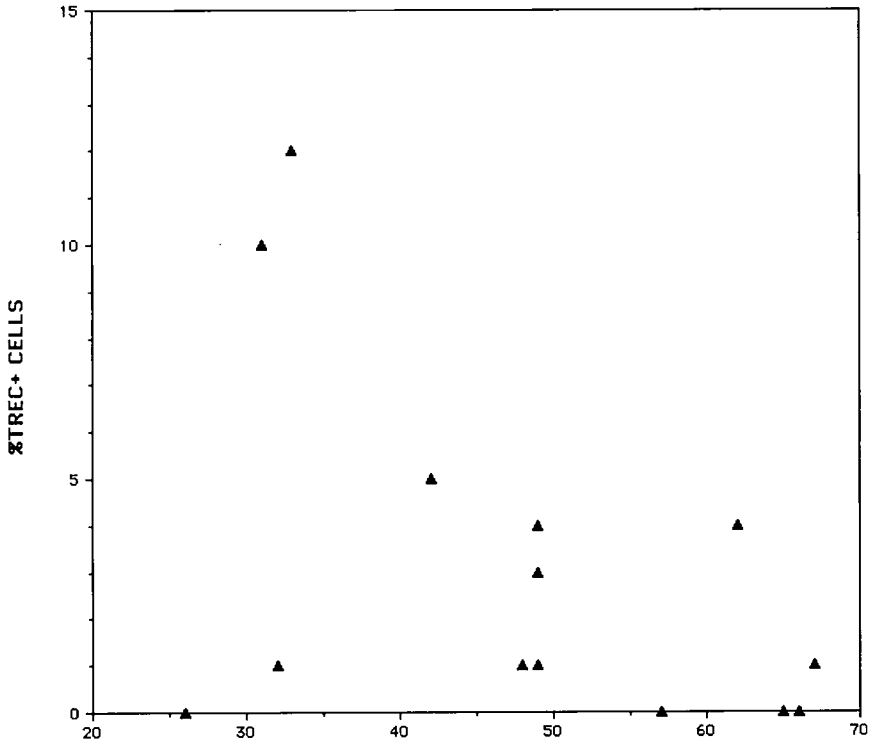
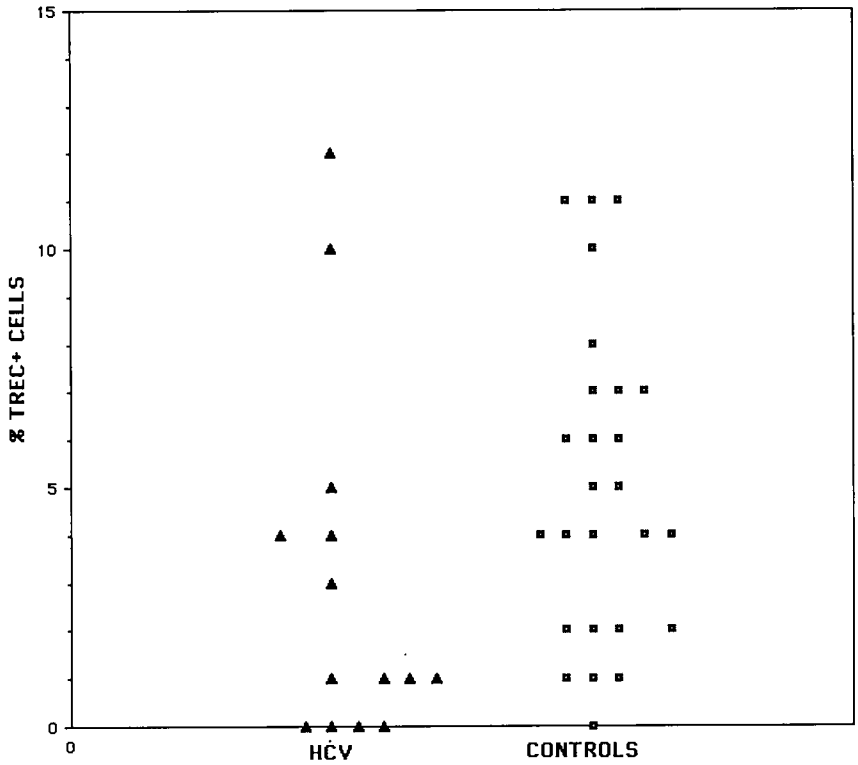


Fig. 2. *Percentages of cj-TREC-positive lymphocytes in relation to age in patients with HCV infection who received no previous treatment.*

Fig. 3. *Percentages of cj-TREC-positive lymphocytes in 14 patients with HCV infection and in the control group*



in Figure 3. Mean percentage was 3.0% for HCV patients and 5.6% for controls. The difference resulted significant (P=0.01) at the *Wilcoxon* signed rank.

DISCUSSION

In this communication, we provide evidence for a significant reduction of RTE in patients with HCV infection, as compared to normal controls of comparable age. To our knowledge this is the first demonstration of an impaired thymic function in HCV infection. Considerable information has been obtained on the role of cytotoxic T-cells in HCV infection. Accapezzato *et al* have shown that patients with chronic HCV infection show concomitant and considerable intra-hepatic expansion of independent subsets of virus-specific CD8+ T cells (11). Others have studied T-lymphocyte responses in index patients with acute HCV and their sexual contacts who developed acute infection and demonstrated that cellular immune responses can develop in exposed but persistently aviremic and antibody-negative individuals (12). However, few data are available on the distribution of cells with naive or memory phenotype in the peripheral blood of infected individuals. A significant higher frequencies of activated memory T-cells (CD45R0+, CD69+) has been reported in patients who had resolved HCV infection, either spontaneously or after anti-viral therapy (13). Taken together, these data support a positive role for T-cells in HCV infected patients.

The best known infectious disease where a defect of the production of TREC+ cells in the peripheral blood has been shown, along with the correlation of RTE percentages with a favorable prognosis, is HIV disease (14). In this case, both the direct cytotoxic effect of HIV on T-lymphocytes and the presence of apoptosis (15) are mechanisms inducing depletion of T-cells in the lack of a sustained thymic output.

However, also in HCV infection, several evidences suggest that T-cells potentially instrumental for inducing a favorable clinical course may be depleted. This is suggested by two different findings. First, Meyer-Olson *et al.* have shown a limitation of the T-cell receptor repertoire that is unable to provide sufficient variability to control the rapidly evolving viral immune evasion (16). In addition, different groups have shown that apoptosis is increased in HCV patients by both activation of apoptotic caspases (17) decreased expression of NF-kappa-B (18) and activation of the Fas-FasL mechanism (19), resulting in a depletion of peripheral lymphocytes. This depletion may be further increased in the lack of an efficient thymic output.

In conclusion, this paper, although limited to the study of cj-TREC, supports the idea of the presence of a thymic defect in early HCV infection. These data add insight in the pathogenesis of ongoing HCV infection. Further studies are in progress to evaluate cj- and sj-TREC in *naive* patients as well as in HCV patients who obtain a viral clearance or cannot clear HCV. This may get insight, at least in part, on the role of thymic defect to the clinical course of this disease.

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