Abstract. – Background/Aims: Cellular immunity has a pivotal role in the pathogenesis of chronic pancreatitis (CP), resulting in pancreas infiltration by T-cells. Studies on systemic immunity are few and contradictory. One study reported a decrease of naive CD45RA+ cells. The presence of naive T cells, detected as recent thymic emigrants (RTEs), is evaluated with a new molecular technique by using real-time PCR to detect the T-cell receptor excision circles (TREC). To elucidate the role of naive T-cells in the pathogenesis of CP, we investigated the percentage of sj-TREC in CP patients.

Patients: Thirty CP patients were studied and compared to 30 sex- and age-matched healthy volunteers.

Methods: Genomic DNA was isolated from peripheral blood mononuclear cells (PBMC) of each patient. RTEs were evaluated by measuring sj-TREC by real-time PCR.

Results: The mean percentage of sj-TREC cells present in CP was not significantly different from that of control group (0.02319% vs 0.02338%, respectively).

Conclusion: Our data show that naive TREC cells are normally represented in CP. The presence of active thymopoiesis may be the underlying mechanism resulting in continuous production of T-cells, responsible of maintaining the inflammatory process.

Key Words:
Chronic Pancreatitis (CP), T-cell Receptor Excision Circles (TREC), Signal joint-TREC (sj-TREC), Recent Thymic Emigrants (RTEs).

Introduction

Chronic pancreatitis (CP) is characterized by painful progressive destruction of the pancreatic gland with loss of its exocrine and endocrine functions. The immunological mechanisms contributing to CP pathogenesis are still poorly known.

Several reports suggested that T-cells are of particular importance. In fact, the pancreas is infiltrated by CD4+ and CD8+ lymphocytes. These data demonstrate a role of cell mediated immunity in CP. Other reports suggest that, apart from local infiltration, CP also induce systemic modifications of the immune system. However, data on the evaluation of peripheral T-cells are anecdotal and limited to cytofluorimetric assays. For instance, Ockenga et al, reported decreased CD8+ cells, while Gansauge et al, in a much larger series of 28 patients, provide evidence of an increase of the same population.

All these data are limited to the 2 main CD4+ and CD8+ populations, while it is well known that cell mediated immunity is under the control of discrete subsets of CD4+ cells, such as memory and naive cells, and their subset, such as central memory (CM) and effector memory (EM). On this field, the data are limited to the pioneer work by Grundsten et al, who showed increase T CM cells and decreased naive T-cells.

In addition, in a disease in which cellular immunity is centrally involved in the pathogenesis, it is somehow surprising that no reports are available on thymic function. This can be explicated in part by the fact that the techniques for such evaluation have become available only recently and involve a detailed molecular analysis not easy to performed. We therefore addressed the questions of evaluating the naive T cells and thymic function with the modern molecular techniques.

With this method, the truly naive T-cells, resulting from thymopoiesis, are detected measuring the T-cell receptor excision circles (TREC)
present in the recent thymic emigrants (RTEs). TREC are DNA fragments representing a by-product 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 moment of the intrathymic maturation of T cells. Since their DNA exists in a circular form, they are not duplicated 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 RTEs. These cells have been proved to be altered in several clinical conditions, with clinical and prognostic implications.

We therefore investigated sj-TREC+ cells in peripheral blood samples from CP patients compared to control subjects.

Material and Methods

Patients
A group of 30 patients with idiopathic CP was prospectively enrolled in the study (21 male patients and 9 female; mean age 48.33 ± 12.64 years. Thirty sex- and age-matched healthy volunteers (21M and 9F; mean age 48.37 ± 13.3 years) were also evaluated. Features of patients, classified according to Cremer’s classification are showed in Tables I, II, and III.

All patients had a history of recurrent pancreatic pain and persistent or recurrent increase of amylase and lipase, 3 patients had recurrent attacks of non-biliary acute pancreatitis and 2 patients had a history of biliary stricture secondary to chronic pancreatitis. None of the patients had abnormally elevated levels of serum gamma globulin and/or IgG or the presence of autoantibodies (such as anti-nuclear antibody, rheumatoid factor, anti-smooth muscle antibody and anti-mitochondrial antibody) suggestive for autoimmune chronic pancreatitis. None of the patients had a alcohol abuse habit (≥ 80 g/die). Anatomical abnormalities were excluded in all cases.

None of the patients had neither acute pancreatitis nor cholangitis at the moment of blood collection.

All CP patients underwent magnetic resonance cholangiopancreatography (MRCP) and/or endoscopy retrograde cholangio-pancreatography (ERCP). Seven out of 30 patients (23.4%) had type I chronic pancreatitis, 6 (20%) a type II, 4 (13.3%) a type III, 10 (33.3%) a type IV and 3 (10%) a type V. None of the patients with chronic pancreatitis had previous neoplastic diseases, had symptoms suggestive of acute inflammatory diseases. Patients were not taking drugs affecting the immune system. All CP patients had a normal blood cells count.

The control group was represented by healthy volunteers who agreed in the participation to the study protocol. None of the volunteers had history of chronic or neoplastic diseases, symptoms suggestive of acute inflammatory diseases and was not taking drugs affecting the immune system. All healthy volunteers had a normal blood cells count.

The study was approved by local Ethical Committee.

Table I. Features of patients with type I chronic pancreatitis according to Cremer’s classification.

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<th>Pancreatic pain</th>
<th>Alcohol abuse (≥ 80 g/die)</th>
<th>Recurrent non-biliary acute pancreatitis</th>
<th>Weight loss</th>
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PCR Assay
Peripheral blood mononuclear cells (PBMC) were separated from 10 mL freshly collected blood, according to standard procedures. Genomic DNA was extracted from $5 \times 10^6$ PBMC, using standard procedures. RTEs were evaluated by measuring sj-TREC in the total T cell population using real time-PCR\textsuperscript{15}. It is performed by two different PCR to quantify sj-TREC and nuclear DNA, required to obtain the number of cells present in the sample.

To quantify sj-TREC, it is used the gold mix (Roche, Monza, Italy) 5X, the primers used at the concentration of 5 microM, were: sj-TREC DIR 5’-CAC ATC CCT TTC AAC CAT GCT-3’ and sj-TREC REV 5’-GCC AGC TGC AGG GTT TAG G-3’ (Tib Molbiol, Genova, Italy). TaqMan probe for sj-TREC, 5’-FAM-ACA CCT CTG GTT GTA AAG GTG CCC ACT-BlackHole-3’, was included at the concentration of 1 microM. One cycle of denaturation was performed (95°C for 10'), followed by 2 turns of amplification (first: 15 cy-

### Table II. Features of patients with type II, III and IV chronic pancreatitis according to Cremer’s classification.

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<th>Type of pancreatitis according to Cremer’s classification</th>
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### Table III. Features of patients with type V chronic pancreatitis according to Cremer’s classification.

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<th>Type of pancreatitis according to Cremer’s classification</th>
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cles 94°C for 10'', 63°C 5'', 72°C for 8''; second:
30 cycles 94°C for 10'', 58°C for 5'', 72°C for 8'')
and by one cycle at 40°C for 30''.

To quantify nDNA, the primers, used at the
concentration of 5 microM, were: gen-DIR 5'-
GGC TCT GTG AGG GAT ATA AAG ACA-3'
and gen-REV 5'-CCA ACC ACC CGA GCA
ACT AAT CT-3', designed on FasL gene se-
quence, present in 2 copies in the human
genome (Tib Molbiol, Genova, Italy). The Taq-
Man probe, GenProbe 5'-FAM-CTG TTC GT
TTC CTG CCG GTG C-BlackHole-3', was in-
cluded in the reaction mixture at concentration
of 1 microM. One cycle of denaturation 95°C
for 10') was followed by 40 cycles of amplifi-
cation (95°C for 10'', 56°C for 8'', 72°C for
15'') and by one cycle at 40°C for 30''. PCR
was performed using Light Cycler 3.5 Instru-
ment, Roche, Italy.

The quantification was performed with an
external standard curve created with the same
experimental protocol for the unknown DNA
samples. The standard introduced were 10^7-10^3.
One sample of known concentration (positive
control) had to be included in every run. The analysis was automatically determining the
crossing point for the sample, achieved by a
software algorithm which identifies the first
turning point of the fluorescence curve, which
servs as the crossing point in calculation
method.

The percentage of PBMC containing sj-TREC
in each sample was obtained from the ratio be-
tween the value of sj-TREC and nDNA multi-
plied by 2, because the FasL gene is present in 2
copies in the human genome.

**Statistical Analysis**

Statistical analysis was performed for the en-
tire group of CP patients as compared to con-
trols. In addition, CP patients with different Cre-
mer’s scores were also compared to controls.
Differences were evaluated by both parametric
(Spearman’s Rho) and non parametric tests
(Kruskal Wallis’ test).

**Results**

The RTEs were present at a level comparable
to that of controls in CP patients (mean percent-
age of sj-TREC+ cells was 0.02338% in controls
(range 0.001-0.09) and 0.02319% in CP patients
(range 0-0.099). No statistically significant dif-
ference was observed in the 2 groups.

Although sj-TREC percentages decrease with
age in both CP and controls, no differences were
observed at the Wilcoxon’s test (Figure 1). The
means of sj-TREC+ cells by linear regression
showed not significantly difference in CP patients
and controls, also if the data were adjusted for age.

The mean percentage of sj-TREC+ cells was
0.028% in type I chronic pancreatitis, 0.021% in
type II, 0.013% in type III, 0.030% in type IV
and 0.002% in type V. No correlation was found
between TREC+ values and different types of
pancreatitis classified according to Cremer’s
classification, by both parametric and non-para-
metric tests.

RTEs were present and prescrived to a degree
comparable to that of controls in all groups of CP
patients.

![Figure 1. %TREC+ cells in CP (grey spots) and in controls (black spots).](image-url)
Active thymopoiesis in idiopathic chronic pancreatitis

Discussion

In this paper, we provide evidence that newly generated naive RTEs are normally present in the peripheral blood of patients with CP, suggesting a normal active thymopoiesis. Our data are at variance with previous report\(^7\) that showed decreased numbers of naive T-cells. The difference could be explained by the fact that we used a newer molecular technique able to recognize truly naive RTEs, as compared to cytometric techniques. In addition, it should be point out that the difference observed by Grundsten et al\(^7\) was more evident in CD4\(^+\) naive cells as compared to CD8\(^+\). Due to limitation on the quantity of blood withdrawn imposed by the Ethical Committee, our technique does not allow to discriminate between CD4\(^+\) and CD8\(^+\) cells. Thus, further studies are needed to evaluate RTEs in different T-cell subsets. On the other hand, the presence of normal naive T-cells does not preclude the possibility that central memory might be related to maintenance of the disease\(^7\), once the thymus has been showed to act as a reservoir of newly generated T-cells.

Moreover, IL-7 plasmatic levels were also evaluated in 8 patients by ELISA (human IL-7, BMS237INST, Bender MedSystems GmbH; Vienna, Austria). Our data show no difference between CP patients and normal controls.

The presence of normal active thymopoiesis may be the underlying mechanism resulting in continuous production of T cells, that are responsible at the tissue level to aliment pancreatic inflammation. Indeed, the involvement of systemic immunity appears to be relevant and possibly involved in the pathogenesis, since is not a mere reflection of ongoing pancreatic inflammation. In fact, Grundsten et al.\(^7\) have shown that systemic alteration persists even after surgical removal of the inflamed tissue.

One addition field of investigation, in which data are lacking, is related to the relationship between systemic and local alterations. In this regard may be of interest our observation that 3/3 CP patients in Cremer’s type V had a 10 times decreased values of RTEs. Although the data are limited to a few patients and are not sufficient to reach statistical significance, such a strong decrease may be related to the fact that in type V, the active inflammatory process is no large present. In alternative, it is possible that the thymus is unable to release enough RTEs to compensate the fact that many T-cells are removed from systemic circulation because of their homing in inflamed pancreas due to the obstructive stenosis of the head. Further studies are needed to correlate TREC analysis with the presence of disease specific genes, such as SPINK-1\(^10\).

References


