HCV RNA SEQUENCES IN EOSINOPHILS OF CHRONIC HCV-INFECTED PATIENTS

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Abstract

In the present study we examined the presence of Hepatitis C virus (HCV) RNA sequences in eosinophils (Eos) isolated from 10 chronically HCV-infected patients. At the time of the study patients showed levels of alanine aminotransferase (ALT) and aspartate aminotranferase (AST) above the normal upper limits (129 IU/L ± 77 and 56.2 IU/L ± 40, respectively). Absolute and relative total leukocyte and Eos counts were within the normal range. Highly purified eosinophils (> 98 %) were obtained by Ficoll-Hypaque (d = 1.114 g/mL) and Percoll gradient centrifugation following by immunomagnetic absorbtion to cell specific antibodies. Mononuclear cells (MN) and neutrophils (Neu) were also purified from these patients. PCR analysis of these cell populations revealed the presence HCV RNA sequences in 4/10 Eos, 6/10 MN and 2/10 Neu cell samples. The results suggest that, in addition to MN and Neo cells, Eos might also be susceptible to HCV infection.

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Introduction

Hepatitis C virus (HCV) is a major cause of chronic liver disease worldwide and it is an important risk factor for the development of hepatocellular carcinoma (Bonino, Oliveri *et al.,* 1995). The HCV is considered a new member of the *Flaviviridae* family. Its genome is a 9.4 Kb single-stranded positive RNA that encodes a large polyprotein, which, after cleavage by cellular and viral proteases, generates several structural and non-structural proteins (Major and Feinstone, 1997). The mechanisms of hepatocellular injury and disease progression in HCV infection are not well understood. It has been proposed that factors such as a lack of an effective immune response, generation of viral mutants (quasispecies), and infection of extra hepatic tissues might contribute to the physiopathological events that lead to the chronic stage of the infection (Corado, Toro *et al.,* 1997; Farci, Alter *et al.,* 1992; Mizokami, Gojobori *et al.,* 1994; Zignego, Maccha *et al.,* 1992). Several authors have reported the capacity of HCV to infect extra hepatic tissues such as the peripheral blood mononuclear cells (PBMC), the second major target cells susceptible to viral infection (Müller, Pfaff *et al.,* 1993; Zignego, Maccha *et al.,* 1992). Recently, we reported an increased H_2O_2 production by polymorphonuclear cells (PMN) of HCV-infected patients. PCR analysis of highly purified PMN cells from these patients revealed the presence of HCV RNA in 27% cell samples (Toro, Conesa *et al.,* 1998). This PMN population contained 98% neutrophils (Neu) as determined by CD16b cell surface expression.

Another important PMN cell subset is represented by the eosinophils (Eos). They are non-dividing, bone marrow-derived, granule-containing cells that circulate in the peripheral blood with a half life of about 18 hours before migrating into tissue (Kita, Adolphson *et al.,* 1998). Eosinophils have a tissue damaging role that contributes to chronic inflammation. They release a number of inflammatory mediators including cytokines, toxic cationic proteins, lipid metabolites and oxygen radicals that may kill or damage infectious organisms and surrounding tissue (Weller, 1994, 1996).

Based on previous and our own reports that showed the capacity of HCV to infect leucocytes, we wondered if eosinophils may also be infected by this virus. We analyzed the presence of HCV RNA sequences in highly purified eosinophil suspensions isolated from HCV-infected patients.

Materials and Methods

Patients

Ten patients (eight males and two females), mean age 40 ± 12.3 years with a diagnosis of HCV chronic infection were studied. All patients were repeatedly positive for antibodies against HCV (anti-HCV) (2nd generation, Ortho Diagnostics, Neckargemünd, Germany), and positive for HCV RNA in serum, as determined by nested PCR according to the method of Inchauspe, Abe *et al.* (1991). None of the patients was at the end stage of liver disease and thus, they were not immunodeficient. They had no evidence of other chronic or autoimmune liver diseases.

Cells Separation

Eos, MN and Neu cell populations were isolated from EDTA anticoagulated blood of 10 HCV-infected patients according to the procedure described previously by Conesa, Pérez *et al.* (1997) with some modifications. Briefly, whole blood (8 mL) was layered on 5 mL Ficoll-Hypaque (d = 1,114 g/mL) and centrifuged at 600 x g for 30 min. In order to obtain the Eos population, the granulocyte layer was collected and washed once with PBS-gel (0.01 M phosphate buffer, 2 mM EDTA, 5 mM glucose and 0.1% gelatin) at 450 x g for 10 min at 4°C. The cells, adjusted to $25–30 \times 10^6/mL$, were centrifuged over a discontinuous isotonic Percoll gradient at 1600 x g for 30 min. Normodense eosinophils from the 1.090 and 1.100 g/mL Percoll layers were collected, washed in PBS gel and the contaminating red cells were lysed with buffered NH_4Cl solution (150 mM NH_4Cl, 10 mM $NaHCO_3$ and 1 mM EDTA). Then, the cell suspension was washed twice with PBS-gel and Eos were further purified by negative selection using immunomagnetic beads. Briefly, the cell pellet was first incubated with mAb anti-CD16 (clone 3G8) for 30 min at 4°C, resuspended in PBS gel-BSA 0.1%, washed twice with PBS gel, and subsequently incubated with the Dynabeads M-450 (Dynal) coated with anti mouse IgG for 20 min at 4°C. CD16 positive cells were immunoabsorbed to the Dynabeads and Eos were recovered on the supernatant.

MN and Neu cell populations were isolated by Ficoll-Hypaque gradient centrifugation and further purified as previously described by Toro, Conesa *et al.* (1998).

Cell Immunophenotyping

Cell immunophenotyping was carried out according to methods previously described (Conesa, Pérez *et al.*, 1997). Cells were incubated with either anti-CD16b-FITC or anti-CD49d-FITC (Immunotech) and analyzed in an Epics Elite flow cytometer (Coulter Electronics, Hialeath, FL, USA), after previous alignment with DNA check fluorescent beads. Mouse IgG1-FITC and IgG1-RD1 (Coulter Immunology, Hialeath, Miami, FL) were used as isotype controls.

HCV-RNA Detection in Eos, Neu and MN Cells

The presence of HCV-RNA in Eos, Neu and MN cell samples was evaluated by nested PCR using specific primers derived from the highly conserved 5' non-coding region of the HCV genome and following the method of Inchauspe, Abe *et al.* (1991). Briefly, after washing four times with PBS, the cells were processed for total RNA isolation using the reagent Trizol™ (Gibco-BRL, Gaithersburg, MD) according to the manufacturer's instructions. For cDNA synthesis, 11 μL of the RNA solution was reverse transcribed in 25 μL reaction mixture containing 20 units of RNasin (Promega, Madison, WI), 100 pmol of specific external anti-sense primer (-324 to -304), reverse transcriptase buffer (50 mM Tris-HCl pH 8.3, 3 mM MgCl2, 75 mM KCl and 10 mM dithiothreitol; Promega), 2 mM each dNTP (Pharmacia, Upsala, Sweden) and 30 units of avian Mieloblastosis virus (AMV) reverse transcriptase (Promega). After overlaying with 30 μL of mineral oil (Sigma, St. Louis, MO) the mixture was initially heated at 65 °C for 4 min and incubated at 43 °C for 60 min. A first round PCR reaction was performed as follows: 10 μL aliquot of the cDNA was added to 40 μL of reaction mixture containing 10 mM Tris HCl (pH 8.5), 50 mM KCL, 3 mM MgCl$_2$, 0.1 % Triton X-100, 1 mM each dNTP, 50 pmol of external sense (-6 to -13) and antisense (-324 to -304) primers and 2 units Taq polymerase (Promega). The reaction was amplified for 35 cycles (95 °C for 1 min, 55 °C for 2 min, 72 °C for 3 min) in a DNA thermal cycler (Perkin-Elmer Cetus Norwalk, CT). For the second round of PCR, one tenth volume of the first PCR round was added to 45 μL of a second reaction mixture that contained the internal sense (-308 to -281) and antisense (-48 to -32) primers. Amplification conditions for the second PCR were the same as those used for the first PCR. A 10 μL aliquot of PCR products was electrophoresed on 3% agarose gel and visualized under ultraviolet light

after staining with ethidium bromide. The expected size of the amplified DNA sequence (267 bp) was assessed by direct comparison with molecular weight markers.

Results

Characteristics of HCV-Infected Patients

Table I shows the clinical and hematological profile of the patients studied. All of them showed a chronic condition with variable degrees of liver damage. Mean levels of alanine aminotransferase (ALT) and aspartate aminotranferase (AST) in these patients were 129 IU/L ± 77 and 56.2 IU/L ± 40, respectively. Eight out of ten patients showed ALT levels significantly higher than the normal range (0-40 IU/L) and only five out of ten showed increased AST levels. Overall, total and relative leukocyte counts were within the normal range. Only one patient showed a total leukocyte number below 4,800 cell/μL. Mean values of relative and absolute Eos counts were 2.9 ± 1.9% and 192 ± 137 Eos/mL, respectively, (normal range, <5% and <400 Eos/mL, respectively).

HCV-RNA Detection in Eos of HCV-Infected Patients

The presence of HCV RNA sequences in Eos of HCV-infected patients was explored by the RT-PCR method. Purity of the Eos preparations was analysed by both differential cell count on preparations stained with eosin/methylene blue solution. The expression of CD16b and CD49d was determined by flow cytometry analysis. Cell preparations were constituted by 98% of eosinophils. Figure 1 shows that 99.5% of the cells expressed CD49d and only 0.3% expressed CD16b.

Figure 2 shows the results of PCR analysis done in Eos, MN, and Neu (CD16b+) cells obtained from three HCV-infected patients. Viral RNA could be detected in the three cell populations of patient 2, MN and Eos of patient 5 and MN cells of patient 10. The presence of serum HCV RNA during cell isolation was excluded by the absence of viral RNA in the third wash of the cells (Figure 2, CW). Table II summarizes the results of this analysis in the ten patients studied. In general, viral RNA was detected in four (40%) Eos, six (60%) MN and two (20%) Neu cell samples. Those patients who showed positiveness for HCV RNA in Eos and/or Neu cells were also positive for this viral marker in their MN cells. However, two of the patients positive for HCV RNA in Eos, were negative in their Neu cells. Four of the patients were negative for viral RNA in all the cell populations studied.

TABLE I
Clinical and Hematological Profile of HCV-Infected Patients

Patient	Liver Biopsy	AST IU/L	ALT IU/L	Total Leuco.	Neu (%)	Ly (%)	Mon. (%)	Eos (%)
1	CH(m)	104.4	236.7	6700	40	57	1	2
2	CH(l)	31	42	5000	50	42.5	5.5	2
3	CH(l)	86	223	4600	53	44	2	1
4	CH(m)	151	189	7700	65	34	0	1
5	nd	18	13	8200	75	20	0	5
6	CH(l)	43	119	6600	31	57	8	4
7	CH(l)	46	187	7300	46	40	9	5
8	CH(l)	77	127	6200	62	37	0	1
9	CH(m)	45	94	6300	38	55	5	2
10	nd	55	68	5600	56	31	6	6

The Table represents the histological, biochemical and haematological profile of the patients studied. The leucocyte counts were determined by an haematological counter (MD, Coulter Corporation) and represent the percentage of identified populations. All the patients were positive for HCV-RNA in serum. The abbreviations are: CH: chronic hepatitis with (l) low activity or (m) mild activity, Neu: neutrophils, Ly: lymphocytes, Mon: monocytes, Eos: eosinophils and nd: not determined.
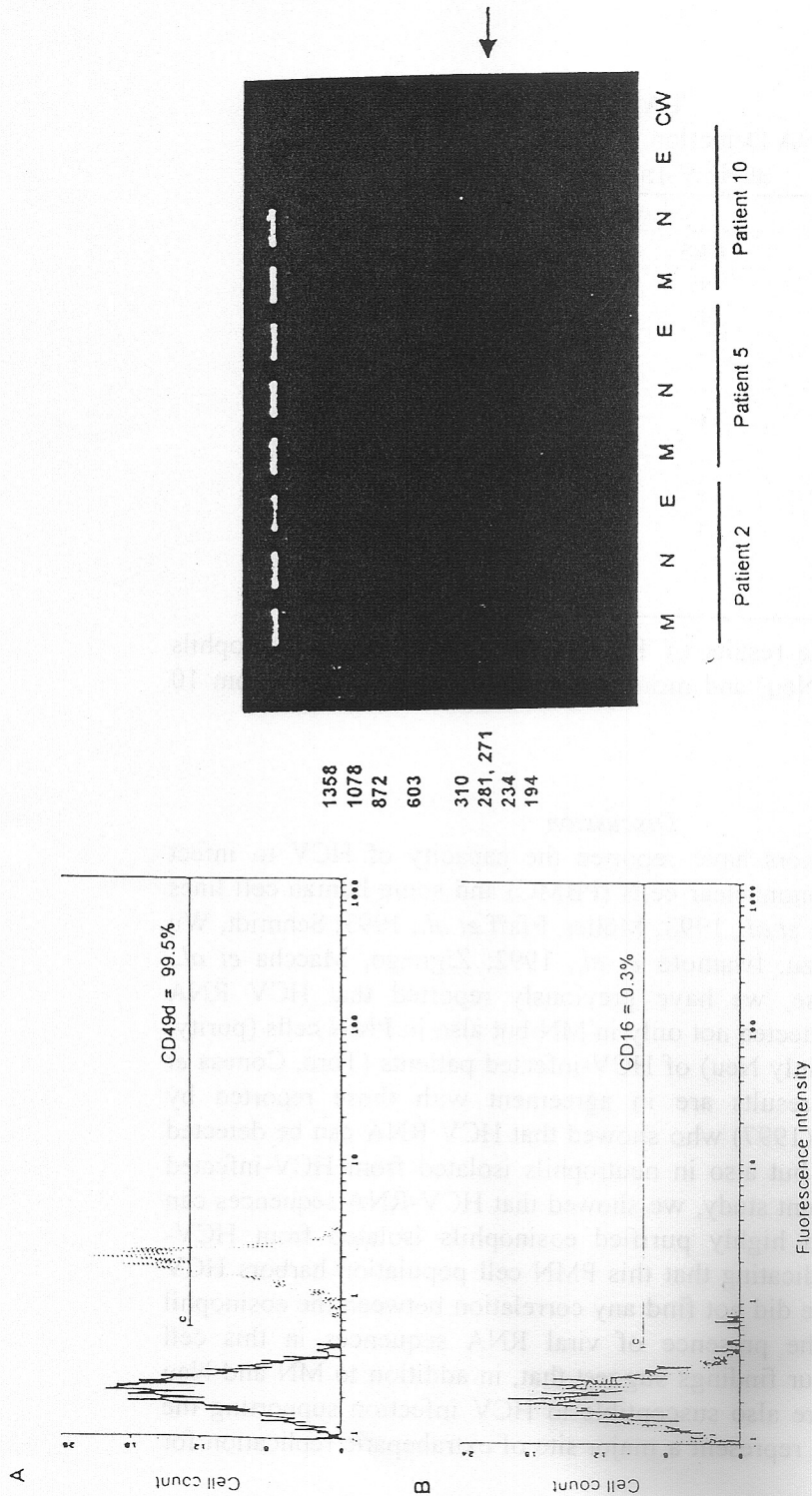
Figure 1.: (Left). Typical histograms showing the immunophenotype of an eosinophil-enriched cell population labeled with (Part A) anti-CD49d and (Part B) anti-CD16b antibodies. The percentage of positive cells (cursor c) was determined by subtracting the isotype control on the two parameters measured.

Figure 2.: (Right). Electrophoretic analysis of PCR products from MN, PMN and Eo. cells of HCV-infected patients. PCR products representing positive strands of HCV-RNA were identified in mononuclear (M), neutrophils (N) and eosinophils (E) isolated from EDTA-anticoagulated blood of HCV-infected patients as described in the Material and Methods section. Figure represents the RT-PCR analysis done in three patients. CW: last wash of eosinophils form patient 2. Molecular weight markers, θX174 DNA digest with *Hae* III, are depicted. The arrow shows the 267 base pair (bp) PCR product.

TABLE II
HCV-RNA Detection in Eos, Neu and MN Cells of HCV-Infected Patients

Patient	Leukocyte Population		
	Eos	Neu	MN
1	+	-	+
2	+	+	+
3	-	-	-
4	-	+	+
5	+	-	+
6	-	-	-
7	-	-	-
8	+	-	+
9	-	-	-
10	-	-	+

The table shows the results of RT-PCR analysis done in eosinophils (Eos), neutrophils (Neu) and mononuclear (MN) cells isolated from 10 patients studied.

Discussion

Several authors have reported the capacity of HCV to infect peripheral blood mononuclear cells (PBMC) and some human cell lines (Bertolini, Iacovacci et al., 1993; Müller, Pfaff et al., 1993; Schmidt, Wu et al., 1997; Shimizu, Iwamoto et al., 1992; Zignego, Maccha et al., 1992). In this sense, we have previously reported that HCV RNA sequences can be detected not only in MN but also in PMN cells (purity: >97% CD16b+, mainly Neu) of HCV-infected patients (Toro, Conesa et al., 1998). These results are in agreement with those reported by Schmidt, Wu et al. (1997) who showed that HCV RNA can be detected not only in PBMC but also in neutrophils isolated from HCV-infected patients. In the present study, we showed that HCV-RNA sequences can also be detected in highly purified eosinophils isolated from HCV-infected patients indicating that this PMN cell population harbors HCV RNA sequences. We did not find any correlation between the eosinophil cell number and the presence of viral RNA sequences in this cell population. Thus, our findings suggest that, in addition to MN and Neu cells, eosinophils are also susceptible to HCV infection supporting the idea that leucocytes represent a major site of extrahepatic replication for

HCV. Whether or not this phenomenon has physiopathological implications in eosinophil effector functions that might contribute to the clinical profile of this chronic viral infection remains to be clarified.

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