CLINICAL IMMUNOLOGY AND IMMUNOPATHOLOGY
Vol. 88, No. 2, August, pp. 169–175, 1998
Article No. II984564

Increased Peroxide Production by Polymorphonuclear Cells of Chronic Hepatitis C Virus-Infected Patients

Felix Toro, Angela Conesa, Alexis Garcia, Nicolas E. Bianco, and Juan B. De Sanctis

Institute of Immunology, Faculty of Medicine, Universidad Central de Venezuela, Apartado 50109, Caracas 1050-A, Venezuela

To evaluate the oxidative burst in hepatitis C virus (HCV) infection, intracellular hydrogen peroxide (H_2O_2) production of polymorphonuclear (PMN) cells isolated from 15 chronic HCV-infected patients and 11 controls was assessed by flow cytometry in a time kinetic. Under nonstimulated and phorbol myristate acetate (PMA)-stimulated conditions, H_2O_2 production was higher in HCV-infected patients than in controls ($P < 0.05$) at the time points of 20, 30, and 40 min. A positive correlation between H_2O_2 production by PMA-stimulated cells and serum levels of alanine aminotransferase and aspartate aminotransferase was found in the HCV-infected patients ($r = 0.877$, $P < 0.01$ and $r = 0.9351$, $P < 0.001$, respectively). RT-PCR analysis of purified mononuclear (MN) and PMN cells from HCV-infected patients revealed the presence of HCV RNA in 60% of MN and 27% of PMN cell samples. These results suggest that a functional alteration of PMN cells is manifested in this chronic viral infection which may represent an additional factor in the development of liver lesions.

Key Words: **polymorphonuclear cells; hepatitis C virus infection; peroxide production; flow cytometry.**

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 (1). The mechanisms of hepatocellular injury and disease progression in this viral infection are poorly understood. Thus, evidence for a direct cytopatic effect by HCV is limited. Some studies have shown a correlation between serum levels of HCV RNA and hepatic lobular inflammation (2, 3) but immunohistochemical studies have not shown a direct correlation between hepatic HCV antigen staining and degree of inflammation (4). Several authors have suggested that HCV chronic infection might be associated with an impaired immune response (5, 6). Some specific immunological responses have been shown to be not altered in HCV infection. For instance, T cell responses to nonstructural and structural viral antigens have been reported in both peripheral blood and liver tissue of HCV chronic carriers (reviewed in 7).

Little is known about the innate immune response in HCV infection. In this regard, we have recently described a functional impairment in natural killer (NK) cell spontaneous cytotoxicity in a group of patients with chronic HCV infection which may be attributed either to the stimulation of NK inhibitory receptors or to the effect of suppressive cells and/or soluble factors released by viral-infected cells (8).

Polymorphonuclear (PMN) cells represent another important component of the primary immune response. This leukocyte population plays a critical role in the host defense mechanism against bacterial infections and secretes several cytokines that regulate the process of hematopoiesis as well as inflammatory mechanisms (9, 10). Concerning the bactericidal function, neutrophils employ nonoxidative and oxidative mechanisms. Nonoxidative mechanisms include release of hydrolases, proteases, cationic proteins, and other bactericidal factors, while oxidative mechanisms involve the generation of NADPH oxidase-derived superoxide (O_2^-) and its dismutation product hydrogen peroxide (H_2O_2) as the primary reactive oxygen species (9).

Previous reports have shown that some PMN cell functions are altered in patients with chronic liver disease. These observations include an impaired neutrophil chemotaxis function (11), a diminished phagocytic activity (12), and an altered capacity of PMN cells to produce oxygen-derived free radicals (13, 14). However, it is not clear how PMN functions may be related to the clinical as well as the virological condition of the patient. To address this further, we assessed the oxidative burst of PMN cells derived from chronic HCV-infected patients under nonstimulated (resting) and PMA-stimulated conditions and correlated their functional status with biochemical and virological parameters.

METHODS

Patients

Fifteen patients (7 males and 8 females), mean age 42 ± 18 years, with diagnosis of HCV chronic infection

0090-1229/98 $25.00

were selected for this study from the Outpatient Clinical Unit of the Institute of Immunology. All patients were repeatedly positive for antibodies against HCV (anti-HCV) (second generation; Ortho Diagnostics, Neckargemünd, Germany). They were also positive for HCV RNA in serum as determined by nested PCR according to the method of Inchauspe *et al.* (15). HCV genotype analysis was performed in patients' sera following the method of Okamoto *et al.* (16). 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.

A control group consisting of 11 healthy individuals (6 males and 5 females), mean age 32 ± 6 years, was selected from the laboratory staff of the Institute of Immunology and from voluntary blood donors of the Central University Hospital. They were seronegative for HCV, HBV, HIV, and Chagas disease. They also showed normal levels of aminotransferases (mean levels of alanine (ALT) and aspartate (AST) aminotrans ferases were 39 ± 14 and 21 ± 6 IU/L, respectively) and bilirubin (0.4 ± 0.2 mg/dl).

The protocol was approved by the Institute's Clinical Committee. A written consent was obtained from each patient and control studied.

Cell Separation

Mononuclear (MN) and PMN cell populations were isolated from EDTA-anticoagulated blood of controls and HCV-infected patients by a modified Ficoll–Hypaque gradient method (17). Briefly, whole blood (8 ml) was layered on 5 ml of Ficoll–Hypaque solution (density, 1.114 g/ml) and centrifuged at 600*g* for 30 min. After centrifugation, MN (upper) and PMN (lower) cell layers were collected and washed once with PBS gel (0.01 M phosphate buffer, 2 mM EDTA, 5 mM glucose, and 0.1% gelatin) at 450*g* for 10 min at 4°C. For PCR analysis, further purification of both cell fractions was done by standard Ficoll–Hypaque gradient centrifugation (density, 1.070 g/ml). Contaminating erythrocytes in the PMN cell fraction were lysed by incubating this cell suspension with buffered NH_4Cl solution (150 mM NH_4Cl, 10 mM $NaHCO_3$, and 1 mM EDTA). Cells were thereafter centrifuged at 450*g* for 10 min at 4°C and washed twice with PBS gel. Cell viability was assessed by trypan blue dye exclusion (>97% viable).

Cell Immunophenotyping

The percentages of T cells, B cells, and monocytes were determined in MN and PMN cell preparations by direct staining with anti-CD3–FITC, anti-CD19–FITC, and anti-CD14–FITC (Coulter Corp. Hialeah, FL), respectively. Double labeling with anti-CD16–FITC and anti-CD56–RD1 (3G8 and NKH1, respectively) (Coulter Corp.) was used for NK cell phenotyping. Expression of CD16b–FITC (Leu 11a–; Becton–Dickinson) on PMN cells was used as a marker for neutrophils. Nonspecific binding was determined using irrelevant mouse Ig isotypes IgG1–FITC and IgG1–RD (Coulter Corp.). Flow-cytometry analysis was assessed by an Epics Profile II flow cytometer (Coulter Electronics), using a 488-nm argon laser excitation, previously calibrated with fluorescent beads.

HCV RNA Detection in MN and PMN Cells

The presence of HCV RNA in purified MN and PMN cells was evaluated by nested RT-PCR using specific primers derived from the highly conserved 5′ noncoding region of the HCV genome (15). Briefly, after the cells were washed four times with PBS, they were processed for total RNA isolation using the reagent Trizol (Gibco BRL, Gaithersburg, MD) according to the manufacturer's instructions. Detection of HCV RNA sequences was carried out following the method described by Inchauspe *et al.* (15). PCR products were analyzed by 3% agarose gel electrophoresis 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.

Oxidative-Burst Assessment in PMN

H_2O_2 production by PMN cells was determined by the formation of 2′,7′-dicholorofluorescin from 2′,7′-dichlorofluorescein diacetate (DCFH-DA) according to the procedure described by Davis (18). PMN cell suspensions were adjusted to 2×10^6 cell/ml and labeled with 100 μl of 0.2 mM DCFH-DA for 15 min at 37°C. Flow-cytometric analysis was performed using 488-nm excitation with a green photomultiplier to quantify fluorescence emission. Peroxide production was assessed in nonstimulated (PBS gel) and phorbol 12-myristate acetate (PMA)-stimulated cells (100 ng/ml) at different time points (0, 5, 10, 15, 20, 30, and 40 min). Specificity of the assay was assessed by incubating PMN cells with 5 mM mannose and 1 mM maleimide as described previously (19).

Statistical Analysis

Measurements from control and patient PMN cell preparations were pooled for data analysis. Statistical analysis was performed using Student's t test for paired and unpaired data and the linear correlation test. The limit for significance was taken as $P < 0.05$.

RESULTS

Characteristics of HCV-Infected Patients

Table 1 shows the clinical, hematological, and virological profiles of the patients studied. All of them showed a chronic condition with variable degrees of liver damage. Only one patient was at the cirrhotic stage. Mean levels of ALT and AST in these patients were 144 ± 97 and 85 ± 78 IU/L, respectively. All the patients were positive for HCV RNA in serum as determined by PCR. Genotype analysis in 12 serum samples showed a prevalence of 100% of the HCV genotype II (1b). Overall, hematological profile was within the normal range. However, some patients presented alterations in their total leukocyte count: 5 of 15 below 4800 cells/μl and only 1 above 10,800 cells/μl. Relative leukocyte count in all the patients was within the normal range.

HCV RNA Detection in MN and PMN Cells of HCV-Infected Patients

Presence of HCV RNA sequences in MN and PMN cells of HCV-infected patients was explored by the RT-PCR method. Purity of these cell preparations was higher than 95% as confirmed by flow-cytometry analysis (Table 2). As shown in Fig. 1, viral RNA could be detected in both MN and PMN cells of some patients. Presence of serum HCV RNA during cell isolation was excluded by the absence of viral RNA in the third wash of the cells (Fig. 1, CW). PCR analysis allowed the detection of viral RNA sequences in at least 10^5 cells. Of the 15 patients studied, HCV RNA was detected in 9 (60%) MN cell samples and 4 (27%) PMN cell samples. Those patients who showed positivity for HCV RNA in PMN cells were also positive for this viral marker in MN cells. Only 1 patient showed a weaker PCR signal in his MN cells compared to PMN cells (Fig. 1, patient C).

Oxidative Burst in PMN Cells

The time course of the oxidative burst of PMN cells isolated from HCV-infected patients and healthy donors was analyzed under nonstimulated and PMA-stimulated conditions. PMN cells from both groups spontaneously produced increasing amounts of H_2O_2 without exogenous stimulus (Fig. 2A). This response was higher in patients compared to controls and significant ($P < 0.05$) at time points of 20, 30, and 40 min. When PMN cells were stimulated with PMA (100 ng/ml), the H_2O_2 production increased significantly ($P < 0.01$) in both groups compared to nonstimulated cells at any point of the kinetic. However, PMA-stimulated cells of HCV-infected patients showed a higher H_2O_2 production compared to controls which was significant from the time point of 20 min onward ($P < 0.05$) (Fig. 2B). Despite a marked increase in H_2O_2 production by PMN of HCV-infected patients, the ratio of PMA-stimulated cell vs basal activity was similar in both patients and controls ($P > 0.05$) at any time point (Table 3).

No differences in H_2O_2 production were observed between patients with chronic hepatitis undergoing low hepatic activity and those with mild activity. When comparing the oxidative-burst response of HCV-infected patients with biochemical and virological parameters, we found a positive correlation between H_2O_2 production of PMA-stimulated cells (time point of 30 min) and the levels of aminotransferases, ALT ($r = 0.88$, $P < 0.01$) and AST ($r = 0.93$, $P < 0.0001$) (Fig. 3). No correlation was seen between these parameters in the control group (ALT, $r = -0.057$ and AST, $r = -0.027$). On the other hand, no correlation was observed between H_2O_2 production and total leukocyte or relative or absolute PMN counts of HCV-infected patients. Moreover, no differences in H_2O_2 generation were seen between those patients whose PMN cells were positive for HCV RNA and those who were negative for this viral marker.

DISCUSSION

PMN cells represent a subset of leukocytes that plays an important role in the primary immune response against bacterial infections (20). Previous reports have shown an impairment of PMN function in chronic liver diseases. These functional defects include a failure in neutrophil chemotaxis (11), a diminished phagocytic activity (12), and an altered capacity of PMN cells to produce oxygen-derived free radicals (13, 14).

One major mechanism exerted by PMN cells in host defense is the generation of microbicidal oxidants. Production of oxygen-derived free radicals by phagocyte NADPH oxidase is a crucial step in this mechanism which can be studied *in vitro* by several methods using activators such as formylmethionylleucylphenylalanine (FMLP) and PMA (20). With regard to viral-mediated diseases, a previous report has shown that PMN oxidative responsiveness following FMLP stimulation seems to be depressed in patients with chronic viral hepatitis at variable disease stages (13). In addition, Giannelli *et al.* (14) have reported that PMA-stimulated PMN cells from chronic HCV-infected patients show a normal production of superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) compared to controls, whereas a diminished O_2^- production is observed only in patients' adherent PMN cells in comparison to controls. These findings point to a selective dysfunction of PMN oxidative response in viral hepatitis. However, it is still not clear how this cellular function is related

TABLE 1

Clinical, Hematological, and Virological Profiles of HCV-Infected Patients

Patient	Liver biopsy	AST IU/L	ALT IU/L	Total leuko	Gr (%)	Ly (%)	Mon (%)	HCV genotype[a]
1	CH(m)	34	97	4,500	74.3	20.4	5.3	II
2	CH(l)	19	22	8,900	53	40.5	6.5	II
3	CH(l)	43	119	6,400	47.3	44.7	8	nd
4	CH(l)	17	17	11,800	63	29.3	7.7	II
5	nd	275	287	6,200	51	49	0	II
6	CH(m)	213	268	3,500	54.3	36.8	8.9	II
7	CH(l)	129	224	2,800	54.9	34.7	10.4	nd
8	Cir	72	111	5,400	54.8	43.2	2	II
9	CH(m)	104	237	6,700	61.3	31.2	7.5	II
10	CH(l)	31	42	5,000	50	42.5	7.5	II
11	CH(l)	86	223	4,600	48.8	43.6	7.8	II
12	CH(l)	24	25	2,500	44.2	46	9.8	nd
13	nd	55	68	5,600	62	31	7	II
14	CH(l)	15	10	5,000	50	49	1	II
15	CH(m)	151	189	7,700	66	34	0	II

Note. The histological, biochemical, hematological, and virological profiles of the patients studied are represented. The leukocyte counts were determined by a hematological counter (MD, Coulter Corp.) and represent the percentage of identified populations. The virological profile was assessed as described under Methods. All patients were positive for HCV RNA in serum. Cir, cirrhosis; CH, chronic hepatitis with (l) low or (m) mild activity; Gr, granulocytes; Ly, lymphocytes; Mon, monocytes; nd, not determined.

[a] HCV genotype was determined according to Okamoto's method.

to clinical and virological characteristics of patients with chronic HCV infection.

In the present report, we evaluated the oxidative burst of highly purified PMN cells derived from chronic HCV-infected patients and related this function to clinical and virological parameters. The results showed that, under both nonstimulated and PMA-stimulated conditions, H_2O_2 production by PMN cells of HCV-infected patients was higher than homologous cells from controls. These results indicate that a priming state of PMN cells is manifested in chronically infected HCV patients.

The oxidative response observed in the HCV-infected patients was independent of the liver histological stage since no differences in H_2O_2 production was detected between patients that showed a low, mild, or severe liver condition (data not shown). However, we found a positive correlation between the H_2O_2 production by PMA-stimulated PMN cells and the levels of aminotransferases in these patients, suggesting that PMN oxidative burst in HCV-infected patients may be a parameter associated with liver function. No correlation with any other biochemical or hematological parameter was observed.

Our finding of an augmented H_2O_2 production by PMA-stimulated PMN cells of HCV-infected patients differs from findings of studies done by Giannelli *et al.*

TABLE 2

Immunophenotype of Mononuclear and Polymorphonuclear Cells Isolated from Peripheral Blood

CD marker	Mononuclear cells	Polymorphonuclear cells
$CD3^+$	80 ± 5	3 ± 1
$CD19^+$	14 ± 6	0.5 ± 0.2
$CD14^+$	4 ± 1	1 ± 0.5
$CD16a^+$ $CD56^{+a}$	8 ± 3	2 ± 1
$CD16b^+$	—	97 ± 2

Note. The means ± standard deviation of the positivity recorded for the 11 samples analyzed using flow cytometry of the purified cell populations as described under Methods.

[a] CD16a/CD56 indicates the positivity recorded for the NK cell population.

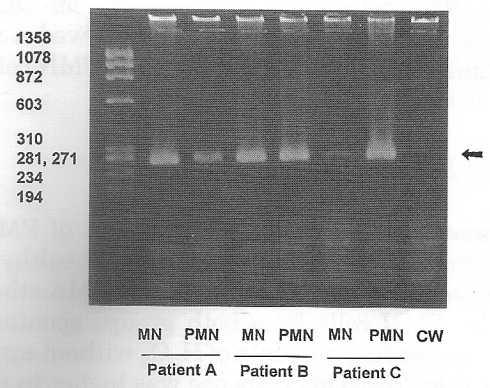

FIG. 1. Electrophoretic analysis of PCR products from MN and PMN cells of HCV-infected patients. PCR products representing positive strands of HCV RNA were identified in MN and PMN isolated from EDTA-anticoagulated blood of HCV-infected patients as described under Methods. The RT-PCR analysis performed in three patients is shown. CW, last wash of PMN from patient C. Molecular weight markers, ϕX174 DNA digest with *Hae*III, are depicted. The arrow shows the 267-bp PCR product.

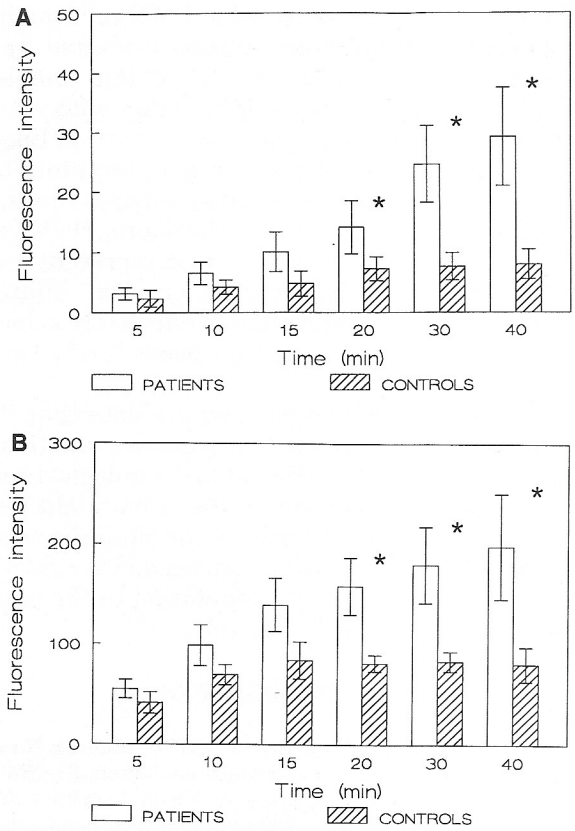

FIG. 2. Kinetics of H_2O_2 production by polymorphonuclear cells. Freshly isolated polymorphonuclear cells from HCV-infected patients and healthy donors were incubated in the absence (A) or presence (B) of PMA: H_2O_2 production was determined by flow cytometry using 2′,7′DCFH-DA as indicated under Methods. Results are presented as $X \pm$ SE of the mean channel fluorescence intensity, expressed in logarithmic units.

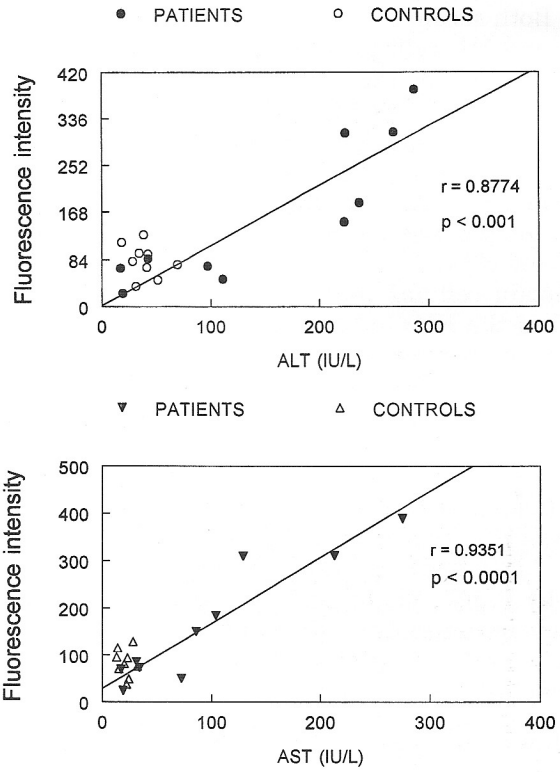

FIG. 3. Correlation between H_2O_2 production and serum levels of aminotransferases in HCV-infected patients. H_2O_2 production by PMA-stimulated PMN cells, measured as the fluorescence intensity of 2′,7′DCFH-DA at the time kinetic point of 30 min, was correlated with the ALT (top) and AST (bottom) serum levels of HCV-infected patients. The straight lines represent the correlation observed in HCV-infected patients. No correlation was observed in the controls.

(14) who reported a normal H_2O_2 generation by suspended PMN cells from chronically HCV-infected patients. Differences in the methodology used to isolate PMN cells and to evaluate H_2O_2 production probably account for these discrepancies. For instance, in our study we measured intracellular H_2O_2 production by flow cytometry using DCFH-DA as probe in contrast to the assay followed by Gianelli *et al.* (14) based on the horseradish peroxidase-mediated oxidation of phenol red by H_2O_2. Thus, estimation of peroxides may vary depending on the technique employed as has been reported by Tsukamoto *et al.* (21).

TABLE 3
Oxidative-Burst Index

Time (min)	Ratio	
	Patients	Controls
5	17.5 ± 2.6	10.2 ± 0.7
10	17.1 ± 3.6	28.2 ± 8.1
15	19.9 ± 4.9	37.5 ± 12
20	23.0 ± 7.4	19.9 ± 4.9
30	23.0 ± 9.6	21.4 ± 7.2
40	11.9 ± 3.4	15.7 ± 6.9

Note. The means ± standard error of the oxidative-burst index of polymorphonuclear cells from HCV-infected patients and healthy donors. This index is the ratio of the baseline to stimulated value of H_2O_2 production at each point of the time kinetic.

A second argument that could explain the augmented oxidative response observed in our patients might be associated with a priming condition manifested in PMN cells due to a cytokine modulator effect. In this regard, elevated serum levels of IL-1, IL-6, and TNFα have been reported in patients with acute and chronic viral hepatitis (22–24). These cytokines play an important role in the process of inflammation and fibrogenesis that is manifested in chronic liver diseases. Among these soluble mediators, special interest has been focused on TNFα because of its pleiotropic properties which include modulation of the immune response to infectious agents and direct antiviral effects

(25). Both serum TNFα and TNFα mRNA levels in the liver and MN cells have been reported to be increased in HCV infection (26, 27). This cytokine has also been involved in PMN functional activity and could be relevant in the regulation of oxidative responsiveness by these cells (20). Thus, it would be interesting to evaluate in detail the role of TNFα as well as other cytokines in the regulation of the oxidative response in HCV infection.

Certain cellular immune responses seem to be altered in the HCV infection. For instance, Jirillo *et al.* (12) have reported that phagocytosis and killing capacity of both PMN cells and monocytes are profoundly depressed in this viral infection. Similarly, we have recently described a functional impairment in natural killer cell spontaneous cytotoxicity in a group of HCV-infected patients (8). This failure in the cytotoxic activity was not related to a deficit in the circulating NK cells. Likewise, our finding of increased H_2O_2 production by PMN cells of HCV-infected patients does not seem to be associated with a differential number of this cell population since the relative leukocyte count in all the patients examined was comparable to controls.

The significance that an enhanced H_2O_2 production by PMN cells in the physiopathology of HCV infection may have is unknown. It has been postulated that, in addition to causing vascular injury, neutrophils are involved in hepatic tissue damage by attacking the parenchymal cells (10). In this process, reactive oxygen species, released by adherent neutrophils, may participate in inactivating plasma antiproteases and thereby allowing tissue proteases to cause parenchymal cell necrosis (10). This represents an interesting mechanism that requires further confirmation.

Several authors have reported the capacity of HCV to infect peripheral blood mononuclear cells (PBMC) and some human cell lines (28–31). In this regard, we examined whether PMN cells were infected by HCV and if this phenomenon might be related with the oxidative burst observed in these cells. Our data showed that HCV RNA was detected mainly in the MN cell fraction, whereas a small proportion of the patients showed viral RNA sequences in their PMN cells. These results are in agreement with those reported by Schmidt *et al.* (32) who showed that HCV RNA can be detected not only in PBMC but also in neutrophils isolated from HCV-infected patients. Overall, these findings suggest that, in addition to MN cells, PMN cells are also susceptible to HCV infection and support the idea that leukocytes represent a major site of extrahepatic replication of HCV. Presence of HCV RNA sequences in PMN cells does not seem to modify the oxidative response of these cells since no differences in H_2O_2 production were observed between patients whose PMN were positive for HCV RNA and those who were negative for this viral marker.

HCV RNA sequences detected in PMN cells might be the result of a phagocytosis process mediated by the neutrophils. Viral particles or viral–protein complexes could adhere to the cells via interaction with specific cell surface receptors such as Fc receptors or lipoprotein receptors (33, 34). These kinds of receptors have been reported in different leukocyte populations, including PMN cells (35, 19). Furthermore, HCV interaction with these cell receptors could represent a critical step in the initial process of viral infection. Whether active viral replication is effectively achieved in the PMN cells is a subject that needs further investigation.

In summary, we have presented evidence that PMN cells of chronic HCV-infected patients are functionally altered regardless of the clinical and virological condition of the patient. PMN cell dysfunction might represent a cofactor that contributes to the clinical course of the disease. Additional studies are needed to clarify the precise role of this leukocyte population in the physiopathology of chronic HCV infection.

ACKNOWLEDGMENTS

This work was supported in part by CONICIT (Consejo Nacional de Investigaciones Científicas y Tecnológicas) Grants S1-2520 and S1-95-000568 and by the GENIC program. The authors are indebted to Dr. Isaac Blanca and Dr. Leopoldo Deibis for their helpful discussions.

REFERENCES

1. Bonino, F., Oliveri, F., Colombatto, P., Calvo, P. L., and Brunetto, M. R., Hepatitis C virus and liver disease. *Acta Gastro. Enterol. Belg.* **58,** 60–67, 1995.
2. Negro, F., Pacchioni, D., Yohko, S., Miller, R. H., Bussolati, G., Purcell, R. H., and Bonino, F., Detection of intrahepatic replication of hepatitis C virus RNA by *in situ* hybridization and comparison with histopathology. *Proc. Natl. Acad. Sci. USA* **89,** 2247–2251, 1992.
3. Lau, J. Y. N., Davis, G. L., Kniffen, J., Qian, K., Urdea, M. S., Chan, C. S., Mizikami, M., Neuwald, P. D., and Wilber, J. C., Significance of serum hepatitis C virus RNA levels in chronic hepatitis C. *Lancet* **341,** 1501–1504, 1993.
4. Krawczynski, K., Beach, M. J., Bradley, D. W., Kuo, G., Di Bisceglie, A. M., Houghton, M., Reyes, G. R., Kim, J. P., Choo, Q.-L., and Alter, M. J., Hepatitis C virus antigen in hepatocytes: Immunomorphologic detection and identification. *Gastroenterology* **103,** 622–629, 1992.
5. Minutello, M. A., Pileri, P., Unutmaz, D., Censini, S., Kuo, G., Houghton, M., Brunetto, M. R., Bonino, F., and Abrignani, S., Compartmentalization of T lymphocytes to the site of disease: Intrahepatic $CD4^+$ T cells specific for the protein NS4 of hepatitis C virus in patients with chronic hepatitis C. *J. Exp. Med.* **178,** 17–25, 1993.
6. Schupper, H. P., Scheffel, H. J., Aceituno, S., Paglieroni, T., Holland, P. V., and Zeldis, J. B., Peripheral-blood mononuclear cell responses to recombinant hepatitis C virus antigen in patients with chronic hepatitis C. *Hepatology.* **18,** 1055–1060, 1993.
7. Cerny, A., and Chisari, F. V., Immunological aspects of HCV infection. *Intervirology* **37,** 119–125, 1994.

8. Corado, J., Toro, F. I., Rivera, H., Bianco, N. E., Deibis, L., and De Sanctis, J. B., Impairment of NK cytotoxic activity in hepatitis C virus infection. *Clin. Exp. Immunol.* **109,** 451–457, 1997.
9. Domachowske, J. B., and Malech, H. L., Phagocytes. *In* "Clinical Immunology Principles and Practice" (R. R. Rose, Ed.), pp. 392–407, Mosby, St Louis, 1996.
10. Jaeschke, H., and Smith, W., Mechanisms of neutrophil-induced parenchymal cell injury. *J. Leukocyte Biol.* **61,** 647–653, 1997.
11. Wyke, R. J., Yousif-Kadaru, I. A., Rajkovic, A. L., Eddleston, W. F., and Williams, R., Serum stimulatory activity and polymorphonuclear leukocyte movement in patients with fulminant hepatic failure. *Clin. Exp. Immunol.* **50,** 442–449, 1982.
12. Jirillo, E., Greco, B., Caradonna, L., Satalino, R., Pugliese, V., Cozzolongo, R., Cuppone, R., and Manghisi, O. G., Evaluation of cellular immune response and soluble mediators in patients with chronic hepatitis C virus (cHCV) infection. *Immunopharmacol. Immunotoxicol.* **17,** 347–364, 1995.
13. Uehara, M., and Sato, N., Impaired ability of neutrophils to produce oxygen-derived free radicals in patients with chronic liver disease and hepatocellular carcinoma. *Hepatology* **20,** 326–330, 1994.
14. Giannelli, G., Piazzolla, G., Tortorella, C., Jirillo, E., Schiraldi, O., and Antonaci, S., Neutrophil oxidative responsiveness in chronic hepatitis C. *Med. Sci. Res.* **24,** 99–101, 1996.
15. Inchauspe, G., Abe, K., Zebedee, S., Nasoff, M., and Prince, A. M., Use of conserved sequences from hepatitis C virus for the detection of viral RNA in infected sera by polymerase chain reaction. *Hepatology* **14,** 595–600, 1991.
16. Okamoto, H., Sugiyama, Y., Okada, S., Kurai, K., Akahane, Y., Sugai, Y., Tanaka, T., Sato, K., Tsuda, F., Miyakawa, Y., and Mayumi, M., Typing hepatitis C virus by polymerase chain reaction with type specific primers: Application to clinical surveys and tracing infectious sources. *J. Gen. Virol.* **73,** 673–679, 1992.
17. Ferrante, A., and Thong, Y. H., Separation of mononuclear and polymorphonuclear leukocytes from human blood by the one-step Hypaque–Ficoll method is dependent on blood column height. *J. Immunol. Methods* **48,** 81–85, 1982.
18. Davis, B., Oxidative burst methods. *In* "Handbook of Cytometry Methods" (J. P. Robinson, Ed.), pp. 146–154, A. R. Liss, New York, 1993.
19. Lara, L. L., Rivera, H., Perez, P. C., Blanca, I., Bianco, N. E., and De Sanctis, J. B., Low-density lipoprotein receptor expression and function in human polymorphonuclear leukocytes. *Clin. Exp. Immunol.* **107,** 205–212, 1996.
20. Morel, F., Doussiere, J., and Vignais, P. V., The superoxide-generating oxidase of phagocytic cells. Physiological, molecular and pathological aspects. *Eur. J. Biochem.* **201,** 523–546, 1991.
21. Tsukamoto, M. J., Saitoh, N., Gohchi, K., Jimbo, S., Shibui, T., and Moriya, K., Polymorphonuclear leucocyte tests: A comparison of cytochrome c reduction and flow cytometric analysis. *Br. J. Biomed. Sci.* **50,** 60–63, 1993.
22. Muto, Y., Nouri-Aria, K. T., Meager, A., Alexander, G. J. M., Eddleston, A. L. W. F., and Williams, R., Enhanced tumor necrosis factor and interleukin-1 in fulminant hepatic failure. *Lancet* **2,** 72–74, 1988.
23. Khoruts, A., Stahnke, L., McClain, C. J., Logan, G., and Allen, J. I., Circulating tumor necrosis factor, interleukin-1 and interleukin-6 concentrations in chronic alcoholic patients. *Hepatology* **13,** 267–276, 1991.
24. Tilg, H., Wilmer, A., Vogel, W., Herold, M., Nölchen, B., Judmaier, G., and Huber, C., Serum levels of cytokines in chronic liver disease. *Gastroenterology* **103,** 264–274, 1992.
25. Tracey, K. J., and Cerami, A., Tumor necrosis factor: A pleiotropic cytokine and therapeutic target. *Annu. Rev. Med.* **45,** 491–503, 1994.
26. Tilg, H., Vogel, W., Wiedermann, C. J., Shapiro, L., Herold, M., Judmaier, G., and Dinarello, C. A., Circulating interleukin-1 tumor necrosis factor antagonist in liver. *Hepatology* **18,** 1132–1138, 1993.
27. Larrea, E., García, N., Qian, C., Civeira, M. P., and Prieto, J., Tumor necrosis factor α gene expression and the response to interferon in chronic hepatitis C. *Hepatology* **23,** 210–217, 1996.
28. Zignego, A. L., Maccha, D., Monti, M., Thiers, V., Mazzetti, M., Foschi, M., Maggi, E., Romagnani, S., Gentilini, P., and Brechot, C., Infection of peripheral blood mononuclear cells by hepatitis C. *J. Hepatol.* **15,** 382–386, 1992.
29. Müller, H, M., Pfaff, E., Goeser, T., Kallinowski, B., Solbach, C., and Theilman, L., Peripheral blood leucocytes as a possible extrahepatic site for hepatitis C virus replication. *J. Gen. Virol.* **74,** 669–676, 1993.
30. Shimizu, Y. K., Iwamoto, A., Hijikata, M., Purcell, R. H., and Yoshikura, H., Evidence for *in vitro* replication in a human T-cell line. *Proc. Natl. Acad. Sci. USA* **89,** 5477–5481, 1992.
31. Bertolini, L., Iacovacci, S., Ponzetto, A., Gorini, G., Battaglia, M., and Carlini, G., The human bone-marrow-derived cell line CE, susceptible to hepatitis C virus infection. *Res. Virol.* **144,** 281–285, 1993.
32. Schmidt, W. N., Wu, P., Han, J.-Q., Perino, M. J., Labresque, D. R., Stapleton, and J. T., Distribution of hepatitis C virus (HCV) RNA in whole blood and blood cell fractions: Plasma HCV RNA analysis underestimates circulating virus load. *J. Infect. Dis.* **176,** 20–26, 1997.
33. Hijikata, M., Shimizu, Y. K., Kato, H., Iwamoto, A., Shih, J. W., Alter, H. J., Purcell, R. H., and Yoshikura, H., Equilibrium centrifugation studies of hepatitis C virus: Evidence for circulating immune complexes. *J. Virol.* **67,** 1953–1958, 1993.
34. Thomssen, R., Bonk, S., and Thiele, A., Density heterogeneities of hepatitis C virus in human sera due to binding of β-lipoproteins and immunoglobulins. *Med. Microbiol. Immunol.* **182,** 329–334, 1993.
35. Traill, K. N., Huber, L. A., Wick, G., and Jürgens, G., Lipoprotein interactions with T cells: An update. *Immunol. Today* **11,** 411–417, 1990.

Received December 4, 1997; accepted with revision April 13, 1998