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Interaction of immune complexes isolated from hepatitis C virus-infected individuals with human cell lines

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Abstract We investigated the interaction of immune complexes (IC) isolated from hepatitis C virus (HCV)-infected individuals with several cell lines that differentially express Fc receptors, and analyzed viral infection by the presence of HCV RNA sequences. Monocytic (U937 and Monomac-6) and lymphocytic (MOLT-4 and Jurkat) cell lines were incubated with interferon-γ plus phorbol myristate acetate to stimulate the expression of Fc receptors before addition of IC. Cell interaction with IC was monitored by flow cytometry. Positive cell fluorescence was detected in U937 and Monomac-6 cells [mean fluorescence intensity (MFI) 10.56 ± 0.8 and 11.60 ± 0.8, respectively]. Incubation of cells with monoclonal antibodies against Fc receptors for IgG before addition of IC decreased MFI in both cell lines (U937 2.1 ± 0.5, Monomac-6 4.4 ± 0.8, $P < 0.001$), indicating that cell-IC interaction through these receptors was inhibited. In particular, the blockage of FcγRII was responsible for this effect. No binding of IC with either MOLT-4 or Jurkat cell lines was detected, which correlated with a very low Fc receptor expression. HCV RNA sequences were identified in the cells up to 120 h of post incubation with IC. These results suggest that IC can mediate entry of HCV to both U-937 and Monomac-6 cell lines mainly through the FcγRII.

Keywords Cryoglobulins · Hepatitis C virus infection · Fc receptors · Flow cytometry

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Introduction

Hepatitis C virus (HCV) is a major cause of chronic hepatitis worldwide. It is estimated that more than 80% of the people infected with the virus remain persistently infected and approximately 20–40% of these individuals develop cirrhosis with a possible progression to hepatocellular carcinoma [13, 14]. Studies focused on the biology of HCV have been hampered by the lack of an efficient culture system that supports viral replication. Some reports have shown that HCV is able to infect not only hepatic cells but also leukocytes [22, 29, 34, 36]. However, the role that these cells play in the physiopathology of this viral infection is poorly understood.

Little is known about the cellular receptor for HCV and the mechanisms by which the virus enters the cell. Several reports have pointed out that CD81 and lipoprotein receptors may represent candidates for viral receptor. CD81 is a member of the tetraspanin superfamily of proteins that shows specific interaction with the HCV envelope glycoprotein E2 [19, 24]. However, evidence supporting the idea that HCV binds to the target cell through the CD81 receptor is not conclusive. A second candidate for HCV receptor is the low density lipoprotein receptor (LDL-R). This protein belongs to a family of structurally closely related cell-surface receptors, which are involved in the cellular uptake of extracellular ligands, and regulate diverse biological processes, including lipid and vitamin metabolism and cell-surface protease activity [20]. Initial studies by Thomsen et al. [31, 32] and others [2, 25] reported an association between HCV and LDL in human sera, and subsequently demonstrated an interaction between HCV or HCV-LDL complexes with cellular LDL-R. More recently, Scarcelli et al. [26] reported that envelope glycoprotein E2 is able to interact with the human scavenger receptor class B type I (SR-BI) expressed in the human hepatoma cell line Hep G2, proposing that this molecule could represent a novel receptor for HCV.

Another mechanism by which viruses may entry to the cell is antibody-dependent enhancement (ADE) in

which virus-antibody complexes may interact with cells via Fc receptors, allowing virus attachment and uptake. This mechanism has been proposed for different classes of virus including flavivirus [9, 10, 21] and retrovirus [11, 16, 28]. The participation of this type of cellular receptors in HCV infection is unknown. This is particularly interesting since some extrahepatic manifestations associated with B lymphocyte proliferative disorders and increased antibody production are well documented in HCV infection [7].

Mixed cryoglobulinemia represents the main extrahepatic manifestation in HCV infection, which is characterized by polyclonal B cell activation and autoantibody production [7]. This is a systemic vasculitis associated with cold precipitable immunoglobulins in the blood. Immunochemical characterization of cryoglobulins from HCV-infected patients reveals the presence of IgG, IgM and HCV particles [1, 7]. Thus, the generation of virus-antibody immune complexes (IC) represents a phenomenon that might be associated with infection of cells that express Fc receptors for immunoglobulins.

We have investigated the role of Fc receptors in HCV infection, analyzing the interaction of cryoglobulins isolated from HCV-infected patients with several cell lines. Characterization of cryoglobulins and their interaction with cell lines that expresses Fc receptors for IgG was evaluated as well as the capacity of HCV to replicate in infected cells.

Materials and methods

Patients

Patients ($n=23$, 14 males and 9 females, mean age 39 ± 18 years) with diagnosis of chronic HCV infection were selected for this study from the Outpatient Clinical Unit of the Institute of Immunology (Caracas, Venezuela). All patients were repeatedly positive for antibodies against HCV (anti-HCV, 3rd generation, Abbott Laboratories). They were also positive for HCV RNA in serum, as determined by nested PCR. Patients had no evidence of other chronic or autoimmune liver diseases and were not on interferon (IFN) therapy at the time of the study.

A control group consisting of 25 healthy, age- and sex-matched individuals, recruited from voluntary blood donors of the Central University Hospital, was also included.

The research protocol was approved by the Ethical Committee of the Institute. Written consent was obtained from each patient and control studied.

Cryoglobulin isolation and characterization

Cryoglobulins were obtained from a 10-ml plasma sample following the method described by Contreras et al. [4]. Briefly, after 7 days at 4°C, plasma samples were centrifuged at 10,4000 *g* for 30 min at 4°C; the supernatant was discarded and 1 ml cold phosphate-buffered saline (PBS, pH 7.6) was added. After 1 h at 4°C, the tubes were centrifuged at 10,400 *g* for 30 min at 4°C; the supernatant was discarded and the cryoprecipitates were consecutively washed four times with 30 ml distilled water at 10,400 *g* for 30 min, at 4°C. Finally, the cryoglobulins were dissolved in 1 ml cold PBS and stored at 4°C. Before analysis, samples were incubated at 37°C for 1 h to allow resolubilization. Cryoprecipitates were studied as follows: protein content was estimated by spectrophotometry at 280 nm (ϵ % 14.5 for human IgG), the presence of IgG, IgM, IgA, C3 and C4 was determined by nephelometry (Beckman Coulter, Galway, Ireland). Anti-HCV IgG and IgM antibodies were determined by ELISA (3rd generation, Abbott Laboratories) and HCV-RNA by nested PCR.

Circulating IC (CIC) in serum and cryoprecipitate samples were assessed by a standardized microassay based on the method reported by Orozco et al. [23]. Total protein content was estimated by UV spectrophotometry [4]. IgG, IgM and IgA immunoglobulins were determined by nephelometry (Beckman Coulter). HCV-specific IgG was determined by an anti-HCV antibody kit (Abbott Laboratories).

Cell lines and flow cytometry analysis

U-937 (ATCC 1593.2), MOLT-4 (ATCC 1582) and Jurkat (ATCC TIB 152) cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA). Monomac-6 cell line was kindly donated by Dr. Luigi Varesio (National Cancer Institute, USA). Cells were cultured for 18 h. at 37°C, 5% CO_2 with optimal doses of phorbol myristate acetate (PMA, Sigma; 10 ng/ml) and IFN-γ (Sigma; 1 ng/ml) to promote expression of Fc receptors for IgG. Expression of CD64 (FcγRI), CD32 (FcγRII), and CD16 (FcγRIII) was assessed by direct staining with anti-CD64-FITC, anti-CD32-RD and anti-CD16-RD monoclonal antibodies, respectively (Immunotech, Coulter Corporation, Miami, FL). Nonspecific binding was determined using irrelevant mouse IgG isotypes IgG1-FITC and IgG1-RD (Immunotech). Flow cytometry analysis was performed in an Epics Elite flow cytometer (Coulter Electronics, Hialeah, FL), using a 488-nm argon laser excitation.

Cryoglobulins binding assays

To establish optimal conditions for binding assays, we carried out experiments using a pool of cryoglobulins from HCV patients that were positive for HCV RNA. Various concentrations of cells, cryoglobulins and different times of incubation were used to ascertain the optimal conditions. Cells lines (2×10^6 cell/ml) were incubated with 100 µl of pooled cryoglobulins (1 mg/ml) for 30 min at 37°C. After four washes with PBS (pH 7.2), cells were incubated with 2.5 µl (1.25 µg) of $F(ab')_2$ goat anti-human IgG-FITC (Immunotech) for 30 min at 4°C. Following three washes with PBS (pH 7.2), specific fluorescence was analyzed by flow cytometry. Specificity of cryoglobulins binding to FcγR was assessed by blocking the different FcγR subclasses with 20 µl (2 µg) of azide-free monoclonal antibodies to FcγRIII (CD16, kindly donated by Dr. John Ortaldo, National Cancer Institute, USA), FcγRII (CD32) and/or FcγRI (CD64) (Dako, CA) prior to cryoglobulin incubation followed by the aforementioned protocol. The blocking antibodies correspond to clones 3G8 (CD16), KB61 (CD32) and 10.1 (CD64).

Detection of HCV RNA in cells incubated with HCV-positive cryoglobulins

To analyze virus internalization by human cell lines, cells (2×10^6 cell/ml), were incubated under non-stimulated or stimulated conditions (PMA and IFN-γ) with 100 µl HCV-positive cryoglobulins for 18, 40, 60, 80 and 120 h. After incubation, cells were washed four times with PBS (200 g, 10 min) and resuspended in 1 ml of the same buffer. To eliminate nonspecific adherence, cells were treated with a digestion buffer (0.05% trypsin, 5 mg/ml RNase, 0.02% EDTA) for 15 min at 37°C as described previously [5]. After an additional wash with PBS, cells were processed for RNA isolation and PCR analysis.

RNA isolation

Total RNA from serum (100 µl), cryoglobulins (1 ml) or cell lines (2×10^6 cells) was extracted using the reagent TRIzol (GIBCO-BRL, Gaithersburg, MD) according to the instructions of the manufacturer. Isolated RNA was finally resuspended in nuclease-free water (Promega, Madison, WI) and stored at −70°C.

HCV RNA detection

Genomic HCV RNA was detected in cryoglobulins and cell samples by the nested PCR method [15]. A set of oligonucleotide primers derived from the highly conserved 5' non-coding region of the HCV genome were used for cDNA synthesis and PCR amplification. The amplification conditions were the same to those described by Inchauspe et al. [15]. HCV negative-strand RNA detection was carried out in cell samples using an HCV-specific sense oligonucleotide and a thermostable reverse transcriptase kit for cDNA synthesis (Perkin Elmer Roche, Branchburg, NJ). Strand specificity of this reverse transcription (RT)-PCR assay for the detection of negative-strand RNA was achieved by conducting cDNA synthesis at high temperature (70°C) using the thermostable enzyme *Tth*. Additional controls in the assay included inactivation of reverse transcriptase at 94°C for 2 h and cDNA synthesis without RNA template. PCR products were analyzed by 3% agarose gel electrophoresis and visualized under UV light after staining with ethidium bromide. The expected size of the amplified DNA sequence (250 bp) was assessed by direct comparison with molecular weight markers.

Statistical analysis

Measurements from control and patient samples were pooled for data analysis. Statistical analysis was performed using Student's *t* test for unpaired data and the Tukey-Kramer multiple comparison test (ANOVA test). The limit for significance was taken as $P < 0.05$.

Results

Characterization of cryoglobulins from HCV-infected patients

Figure 1 shows the immunochemical characterization of cryoglobulins samples isolated from 23 HCV-infected patients and 25 healthy controls. High concentrations of IgG and IgM were found in the patients' cryoglobulins as compared to controls (Fig. 1A). Moreover, significant differences were observed between immunoglobulin levels in the group of patients where IgG > IgM > IgA ($P < 0.05$). Levels of C3 and C4 in cryoglobulins samples of both patients and controls were below the detection limit of the assay (C3 < 0.90 mg/100 ml and C4 < 0.29 mg/100 ml). CIC in both serum and cryoglobulins were significantly higher in patients than in controls (Fig. 1B). Specific anti-HCV IgG was also identified in 6 out of 23 (26%) cryoglobulins samples, while HCV-RNA was detected in all of the cryoglobulins assayed. A pool of three cryoglobulin samples from HCV-infected patients, all positive for anti-HCV antibodies and HCV RNA, was used in the next set of experiments.

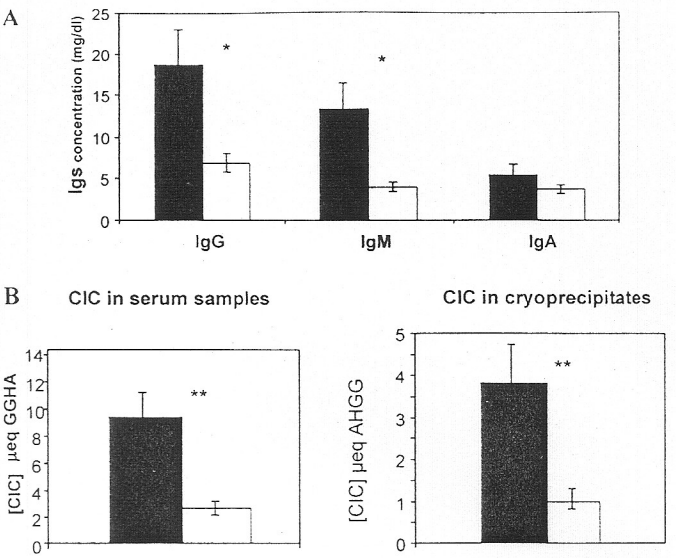


Fig. 1 Immunochemical characterization of cryoglobulins isolated from HCV-infected patients and healthy controls. **A** Levels of IgA, IgG and IgM were determined in cryoglobulins samples by nephelometry as indicated in materials and methods. **B** CIC were determined in serum samples and cryoprecipitates. Values are expressed as µeq of aggregated human gamma globulin (*GGHA*) and represent mean ± SD of 23 patients (*filled bars*) and 25 controls samples (*open bars*). Laboratory references values for immunoglobulins in cryoglobulins were IgG (0–12 mg/100 ml), IgM (0–6 mg/100 ml), IgA (1–7 mg/100 ml). $*P < 0.05$, $**P < 0.01$ (*HCV* hepatitis C virus, *CIC* circulating immune complexes)

Fc receptor expression in cell lines

To establish cell culture conditions for optimal expression of Fc receptor for IgG in cell lines, we measured all three Fc receptors, CD64 (FcγRI), CD32 (FcγRII) and CD16 (FcγRIII) upon stimulation with PMA and IFN-γ. A dose-response curve was previously set up to determine optimal dose of both stimuli. Table 1 shows that, under optimal stimulation conditions (10 ng ml PMA and 1 ng/ml IFN-γ), both U937 and Monomac-6 cell lines exhibited high levels of FcγRII and FcγRI, whereas very low levels of these proteins were detected in Jurkat and MOLT-4 cells lines. In addition, U937 showed significant higher levels of FcγRIII as compared

Table 1 Expression of Fc receptors for IgG in different cell lines. Data represent mean ± SD of three experiments

Cell line	Expression of Fc receptor for IgG (%)		
	CD16	CD32	CD64
U-937	13.3 ± 1.6^a	74.5 ± 3.3^b	81 ± 0.1^b
Monomac-6	1.3 ± 4.6	85.2 ± 2.3^c	66.3 ± 0.2^c
MOLT-4	0.7 ± 0.10	0.8 ± 0.01	0.7 ± 0.06
Jurkat	0.7 ± 0.15	0.5 ± 0.3	8.1 ± 0.4

[a] $P < 0.01$ compared to Monomac 6, MOLT-4 and Jurkat cell lines
[b] $P < 0.001$ compared to Monomac 6, MOLT-4 and Jurkat cell lines
[c] $P < 0.001$ compared to MOLT-4 and Jurkat cell lines

to the other cell lines. When comparing receptor density in non-stimulated U937 cells with stimulated cells, an increase was observed in all Fcγ receptors, particularly in FcγRII [mean fluorescence intensity (MFI) 3.1 ± 0.3 non-stimulated vs 10.2 ± 1.2 stimulated cells, $P < 0.001$]. A similar, although not significant effect was observed in Monomac-6 cells (MFI 2.4 ± 0.3 non-stimulated vs 3.6 ± 0.2 stimulated cells).

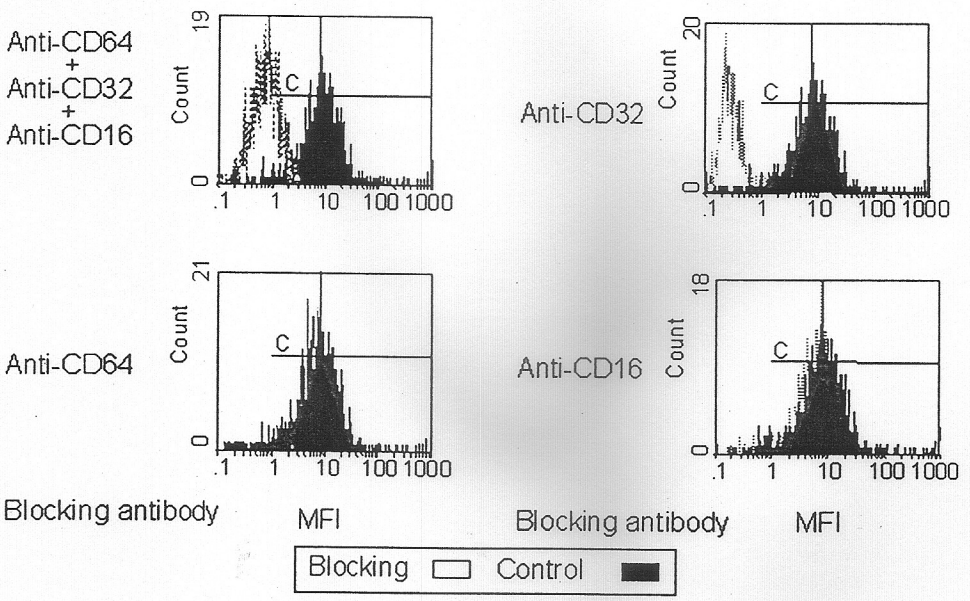
Cryoglobulin binding to cell lines

To evaluate the capacity of cryoglobulin binding to cell lines, U937, Monomac-6, MOLT-4 and Jurkat cells were cultured under non-stimulated or stimulated conditions with PMA and IFN-γ and then incubated with cryoglobulins. Specific interaction was analyzed by flow cytometry after incubation with $F(ab')_2$ goat anti-human IgG-FITC. Figure 2 represents histograms showing the MFI of U937 cells incubated with cryoglobulins (control) or under blocking conditions (cells incubated with anti-Fcγ receptors before cryoglobulins addition). Binding of HCV-positive cryoglobulins to U937 cells was clearly detected (Fig. 2, control condition). Incubation of cells with monoclonal antibodies against FcγR (CD64, C32 and CD16) before addition of cryoglobulins decreased MFI, as compared to the control, indicating that cryoglobulins binding specifically occurred through these receptors (Fig. 2, blocking condition and Table 2). When evaluating the individual effect of each anti-Fc receptor antibody, only the anti-FcγRII antibody showed a marked inhibition of this interaction (Fig. 2, blocking condition and Table 2). Similar results were obtained with Monomac-6 cells (Table 2). In contrast, no binding of HCV-positive cryoglobulins with Jurkat and MOLT-4 cell lines was detected (Table 2).

Fig. 2 Binding analysis of cryoglobulins with cell lines. A typical binding analysis is represented. The U-937 cell line was stimulated by incubation with PMA and IFN-γ and then incubated with cryoglobulins isolated from HCV-infected patients. Specific fluorescence was analyzed by flow cytometry after incubation with $F(ab')_2$ goat anti-human IgG-FITC. Histograms show the MFI for cells under control or blocking conditions (cells incubated with anti-Fc receptors antibodies before addition of cryoglobulins). Total cell count for each experiment was 5,000. The specific positiveness was assessed using the immuno-4 program (Coulter Electronics) (*PMA* phorbol myristate acetate, *MFI* mean fluorescence intensity)

Detection of HCV RNA in U-937 cells incubated with HCV-positive cryoglobulins

To analyze whether interaction of HCV-positive cryoglobulins with cell lines was associated with HCV internalization, we performed RT-PCR analysis to detect HCV RNA in U937 cells incubated with these cryoglobulins. Cells were incubated with cryoglobulins under non-stimulated or PMA- and IFN-γ-stimulated cells. Total RNA was isolated from cells and cryoglobulins samples and then processed for identification of HCV RNA and glyceraldehyde 3-phospahate dehydrogenase (GAPDH) mRNA by RT-PCR. Figure 3 shows the electrophoresis analysis of the PCR products. No HCV-RNA sequences were detected in non-stimulated cells cultured with cryoglobulins (lane 1). However, when cells were stimulated with PMA and IFN-γ and then incubated with HCV-positive cryoglobulins, viral RNA sequences could be identified (lane 2). This positive signal was abolished when cells were either pre-incubated with a set of monoclonal antibodies to Fc receptors for IgG (anti-CD16/32/64) or with the anti-CD32 antibody alone before addition of HCV-positive cryoglobulins (lanes 3 and 4, respectively). No blocking effect was observed with anti-CD16 or anti-CD64 antibodies (lanes 5 and 6, respectively).



these receptors is CD81, a transmembrane protein that may act as direct ligand of the HCV through the virus envelope protein E2 [24]. CD81 is expressed on virtually all nucleated cells, particularly in B cells, where its level of expression is high and varies during development and in response to cellular activation [19]. A second candidate as a HCV receptor is the LDL-R, which may interact with HCV or HCV-LDL complexes [2].

A major feature of HCV infection is the high level of CIC found in patients chronically infected by the virus [12, 18, 35]. This observation has lead to the proposal that CIC may be involved in the pathogenesis of chronic hepatitis C. In fact, a major clinical picture in HCV-infected patients is represented by mixed cryoglobulinemia, a pathology characterized by high levels of cryoglobulins (single or mixed IgG that can reversibly precipitate in cold). The prevalence of mixed cryoglobulinemia in HCV infection varies widely from 13% to 54% (reviewed in [7]), and the levels of monoclonal and polyclonal antibodies as well as of HCV-RNA in the cryoprecipitates are consistently higher than in the corresponding serum. These observations reflect specific interactions between virus and antibodies rather than a merely nonspecific precipitation of cold-insoluble proteins [7]. In this study, we investigated the interaction of IC (cryoprecipitates), isolated from chronically HCV-infected individuals, with several cell lines that differentially express Fc receptors for IgG. The results showed that cryoglobulins containing HCV and immunoglobulins interact with those cell lines that expresses Fc receptors for IgG (U937 and Monomac 6 cells), whereas no interaction was demonstrated with cells that poorly express this kind of receptor, represented by MOLT-4 and Jurkat cell lines. Preincubation of cells with monoclonal antibodies against Fc receptors for IgG (CD64, CD32 and CD16) abolished the binding of IC. This effect was clearly observed with the anti-CD32 monoclonal antibody, suggesting that cryoglobulins binding occurs through the FcγRII receptor. Furthermore, all cells tested express LDL and CD81 receptors: however, not all the cells get infected with cryoglobulins, suggesting that the mechanism of HCV cell entrance is specific for FcγRII.

To further investigate the capacity of virus-immunoglobulin complexes to infect cell lines, we performed RT-PCR assays to analyze the presence of HCV RNA sequences. Viral RNA was identified only in those cells lines (U-937 and Monomac cells) that showed binding of HCV-positive cryoglobulins. No viral RNA sequences were identified in either MOLT-4 or Jurkat cell lines, a finding that correlates with the very low binding activity of IC observed with these cells lines. These results suggest that HCV-immunoglobulin complexes could mediate entry of HCV to target cells that expresses Fc receptors.

Moreover, using a thermostable reverse transcriptase kit, which allows cDNA synthesis at high temperatures (70°C) and thus prevents self-priming of plus-strand HCV RNA sequences, negative strands of HCV RNA were detected in Monomac cells up to 120 h after incubation with cryoprecipitates. This finding supports the hypothesis that viral replication may occur in this cell line.

The finding that FcγRII (CD32) receptor is mainly involved in HCV-immunoglobulin complex binding to cells may be attributed to either its higher expression in both U937 and Monomac-6 cells or that CD32 is a receptor that binds aggregated immunoglobulins or antibodies complexed to multivalent antigens with high avidity [6]. The physiopathological implication of this phenomenon is a matter that needs furthers investigation. It is well known that FcγRII mediates cellular inactivation of B cells when IgG-antigen complexes bind simultaneously to CD32 and B cell receptor on the same B cell. This inhibition effect is dependent on immunoreceptor tyrosine-based inhibitory motifs (ITIM) located in the cytoplasmic segment of CD32 [6]. Thus, a similar event may occur in HCV infection.

Several authors have reported the capacity of HCV to infect peripheral blood mononuclear cells (PBMC) and some human cell lines [3, 22, 29]. Toro et al. [33, 34] reported that PBMC and purified eosinophils from HCV patients were positive for HCV RNA. This phenomenon was associated with a functional alteration in the oxidative burst of these cells [33]. Schmidt et al. [27] also demonstrated that HCV RNA could be detected in PBMC and neutrophils isolated from HCV-infected patients. Thus, HCV RNA sequences detected in PBMC might be the result of phagocytosis mediated by neutrophils. By this mechanism, known as ADE, viral-immunoglobulin complexes could adhere to the cells via interaction with Fc receptor. This mechanism may be involved in monocytes, B lymphocytes and dendritic cells infection [6].

Besides interacting with Fc receptors, IC enhance, both in vitro and in vivo, cytokine transcription and secretion from PBMC and it may trigger the complement cascade [8]. Recently, Kanto et al. [18] reported that IC-type HCV particles could inhibit cytotoxic T lymphocyte responses in vitro by decreasing expression of the costimulatory molecule B7-1 on monocytes and enhancing production of TGF-β1. Thus, HCV-immunoglobulin complexes may have an important role not only in the pathogenesis of extrahepatic syndromes but also in regulating the immune function of the infected patient.

HCV interaction with Fc receptors probably represents a critical step in the initial process of viral infection. Whether or not active viral replication is effectively achieved in those cells that express this kind of receptor is a subject that needs further investigation. Another interesting hypothesis proposes that Fc receptor-expressing cells may harbor virus-immunoglobulin complexes and transfer infectious IC to susceptible cells. Several studies based on the HIV model support this idea [11, 16, 30]. For instance, Heath et al. [11] showed that HIV complexed with neutralizing antibodies was not infectious when incubated with T cells but that the virus-antibody complexes were infectious for T cells when bound to follicular dendritic cells. More recently,

Jakubik et al. [16] reported that B lymphocytes could carry and transfer infectious HIV IC to T cells. Thus, the above studies suggest a novel mode of infection of T cells by HIV that could be important in the pathogenesis of viral infection. Likewise, we propose that HCV-immunoglobulin complexes may interact with cell surface through Fc receptors, leading to viral entry or allowing interaction with other types of cell that are susceptible to infection. Elucidating the characteristics of antibodies that participate in IC formation may contribute to future studies in HCV vaccine design.

In conclusion, this report shows for the first time that HCV-immunoglobulin complexes can bind to cell lines that expresses Fc receptors for IgG, with FcγRII being critical for this interaction, which may lead to viral entry. Further studies are needed to elucidate the precise role of this kind of interaction in the immunopathogenesis of HCV infection.

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