Impairment of natural killer (NK) cytotoxic activity in hepatitis C virus (HCV) infection

J.C. CORADO, F. TORO, H. RIVERA, N. E. BIANCO, L. DEIBIS & J. B. DE SANCTIS Institute of Immunology, Faculty of Medicine, Universidad Central de Venezuela, Caracas, Venezuela

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SUMMARY

In the present study, we evaluated the NK cell cytotoxic activity in a group of HCV-infected individuals. Although the number of NK cells present in the peripheral blood of the HCV-infected patients was comparable to non-infected individuals, spontaneous NK cytotoxicity was four-fold lower ($P < 0.001$) than in normal donors. This functional impairment was not overcome by depletion of adherent or B cells, and it was partially restored by short-term (18 h) stimulation with IL-2. However, long-term stimulation (72 h) with this lymphokine induced activated killer cell (LAK) activity comparable to normal controls. The reduction in NK cytotoxic response does not seem to be due to soluble suppressive factors, since incubation of normal peripheral blood mononuclear cells (PBMC) with infectious HCV sera for a 4-h period does not affect NK spontaneous cytotoxic activity. Successful $in vitro$ infection of PBMC with HCV infectious serum also resulted in an impairment of NK cytotoxicity, suggesting that altered NK function is associated with HCV infection and may be responsible, at least in part, for the chronicity of the infection.

Keywords natural killer cells hepatitis C virus infection cytotoxicity IL-2 lymphokine-activated killer cells

INTRODUCTION

HCV is responsible for a high percentage of chronic hepatitis in infected individuals [1,2]. It has been suggested that this clinical outcome might be associated with an impaired immune response [3–5]. However, T cell response does not seem to be altered, as reported by several authors [6–8]. In the same context, our group has reported that T cell proliferation and IL-2 receptor expression through CD3 and CD28 activating pathways are unaltered in HCV infection [9]. Other effector mechanisms such as NK cell cytotoxicity have been poorly studied in this infectious disease.

NK cells are a subset of lymphocytes distinguishable from T and B lymphocytes by their phenotype (CD3 $^-$, CD16 $^+$, CD56 $^+$), morphology and functional responses [10,11]. These cells represent between 5% and 15% of the mononuclear cell population, but in some organs, as in the liver, up to 45% of the lymphocyte population are NK cells [10–12].

Host defence against tumour cells and spontaneous lysis of virus-infected cells are the most prevalent functional roles attributed to NK cells [10,11]. These responses may be increased by stimulation with interferons-alpha (IFN-$\alpha$), IFN-$\beta$ and IFN-$\gamma$, and IL-2 [10,11]. Upon stimulation with IL-2, even for short periods of time, enhanced NK lytic activity against K562 cells has been observed [10,11]. Exposure of lymphocytes to high amounts of IL-2 (>1000 U/million cells) for 72 h generates lymphokine-activated killer (LAK) cells. LAK cells are able to lyse tumour cells that are resistant to NK cells lysis [10,11]; for example, non-stimulated or short time primed NK cells do not lyse U937 cells effectively, while LAK cells do so efficiently.

There have been several reports of NK cytotoxic impairment upon viral infection [10,11]. Infections by cytomegalovirus, Epstein–Barr virus and herpes virus have been related to low spontaneous NK cytotoxic activity [10,11]. This decrease in cytotoxic response may be a consequence of either viral infection of NK cells themselves or the secretion of soluble factors by infected cells that condition NK spontaneous cytotoxicity.

The aim of the present study was to assess the effect of HCV infection on NK cytotoxicity using as effector cells peripheral blood mononuclear cells (PBMC) from HCV-infected patients and uninfected control PBMC incubated with a HCV RNA-positive serum. The effect of IL-2 on this NK function was also evaluated.
PATIENTS AND METHODS

Patients
Eleven patients (seven males and four females), mean age 45 ± 8 years, with diagnosis of HCV chronic infection were studied. Levels of alanine-aminotransferase (ALT) activity in these patients were above the normal upper limits (128 ± 50 U/l). They showed no evidence of other chronic or autoimmune liver disease. None of the patients was at end-stage liver disease, and thus they were not immunodeficient. All patients were repeatedly positive for antibodies against HCV (anti-HCV) (2nd generation; Ortho Diagnostics, Neckargemünd, Germany). In addition, HCV RNA was assessed in patients’ sera and PBMC by nested polymerase chain reaction (PCR) according to the method of Inchauspe et al. [13], and HCV genotype analysis was determined following the method of Okamoto et al. [14]. Eighteen seronegative healthy individuals were used as controls.

Cell separation
PBMC were separated from heparinized venous blood of healthy controls and HCV-infected patients by standard Ficoll–Hypaque gradient. Cells were adjusted at 2 × 10^6/ml in RPMI 1640–10% fetal calf serum (RPMI–FCS) and separated in four different aliquots: (i) fresh non-treated cells, (ii) cells cultured for 18 h at 37°C without stimulus, (iii) cells cultured for 18 h with 100 U/ml IL-2, (iv) cells cultured for 72 h with 1000 U/ml IL-2 (LAK cell generation).

In order to determine if adherent cells were involved as suppressor cells of NK activity, we re-tested the NK cytotoxic activity in seven out of 11 patients comparing PBMC versus PBMC depleted from adherent cells and B cells. The latter cell population was obtained by sequential incubation of PBMC (5 × 10^6 cell/ml) on plastic surfaces and nylon wool columns. Non-adherent cells (mainly T and NK cells) were incubated in the absence or presence of IL-2, as described above, and tested for NK cytotoxic cell activity.

Cell immunophenotyping
The percentage of T cells, B cells, monocytes and NK cells was determined in whole blood by direct staining with anti-CD3-FITC, anti-CD19-FITC, anti-CD14-FITC (Coulter Corp., Hialeah, FL) and anti-CD16-PE (Leu-11c-PE; Becton Dickinson, Mountain View, CA), respectively. Non-specific binding was determined using irrelevant mouse immunoglobulin isotypes IgG1-FITC, IgG2-FITC and IgG1 RD (Coulter). Flow cytometry analysis was assessed by an Epics 753 flow cytometer (Coulter) using a 488 nm argon laser excitation, previously calibrated with fluorescent beads.

Cytotoxicity assays
A short-term (4 h) chromium release assay, using 51Cr-labelled K562 and U937 cell lines as targets, was performed in order to monitor NK or LAK cytotoxicity, respectively, as described previously [15]. Briefly, 5 × 10^6 K562 or U937 cells (ATTCC, Rockville, MD) were labelled with 150 μCi of Na51CrO4 (200–300 μCi/mmol) (New England Nuclear, Boston, MA) for 1 h at 37°C. Labelled cells were washed three times with RPMI medium plus 5% FCS and resuspended at 5 × 10^6 cells/ml in RPMI containing 10% FCS. A fixed number (5 × 10^3 cells) in 100 μl of labelled K562 or U937 was mixed with 100 μl of effector cells at four different effector-to-target (E:T) ratios (40:1, 20:1, 10:1 and 5:1) in triplicate into 96-well U-bottomed microtest plates (Falcon Plastics, Oxnard, CA). 51Cr release was measured in 100-μl samples of cell-free supernatants using a gamma counter (Compu gamma; Wallac-LKB, Uppsala, Sweden). Total release of radioactivity was determined by counting the radioactivity released from 5 × 10^3 labelled K562 cells treated with 1% Triton-100. The percentage of lysis was calculated by the following formula:

\[
\% \text{ specific lysis} = \frac{\text{Experimental release} - \text{spontaneous release}}{\text{Total release} - \text{spontaneous release}} \times 100
\]

In some experiments, 2 × 10^6 PBMC/ml from normal controls were incubated with 100 μl of HCV RNA-positive serum or with serum from patients with acute hepatitis A (HAV) infection, chronic hepatitis B infection (HBV DNA-positive) or with autoimmune hepatitis (hypergammaglobulinaemia, presence of autoantibodies and absence viral or inherited diseases). Two series of experiments, a short time incubation (4 h) and a long time exposure (48 h), with the sera were performed. At the end of the incubation, cells were washed three times with PBS and cytotoxicity assays were performed as described above.

In another set of experiments, 2 × 10^6 PBMC/ml from three normal controls were infected with HCV RNA-positive serum and used as target for autologous NK cell cytotoxicity. After 48 h of incubation, cells were washed, labelled with Na51CrO4 and used as target in a similar fashion as the K562 cells described above. Non-infected cells were added to the wells at 5:1 and 2:5:1 E:T cell ratios.

In vitro infection of PBMC with HCV RNA-positive sera
PBMC from healthy donors, obtained by Ficoll–Hypaque gradient, were adjusted at 2 × 10^6 cells/ml in RPMI–FCS and then added to the wells of a 24-well culture plate (Falcon, Becton Dickinson). Then, 100 μl of a serial dilution of a HCV RNA-positive serum (genotype II) or control human serum were added to each well and the plate was incubated for 48–96 h at 37°C in a 5% CO2 atmosphere cell incubator. After incubation, cells were collected from the plates washed four times with PBS (200g, 10 min) and resuspended in 1 ml of the same buffer. In order to eliminate non-specific adherence of HCV particles to the cells, PBMC were processed with a digestion buffer following the method described by Cribier et al. [23]. Briefly, cells were incubated with trypsin (final concentration 0-05%) (Gimco-BRL, Gaithersburg, MD), RNase A (5 mg/ml) (Sigma Chemical Co., St Louis, MO) and EDTA (final concentration 0-02%) for 15 min at 37°C. After an additional wash with PBS, cells were processed for total RNA extraction with RNAzol (Biotex, Houston, TX) following the instructions of the manufacturer. Presence of HCV RNA in cell samples and supernatants of the final wash was evaluated by nested PCR according to the method of Inchauspe et al. [13] using specific primers derived from the highly conserved 5’ non-coding region of HCV genome. Additional controls included cells samples incubated with (i) normal human serum (NHS), and (ii) HCV RNA-positive serum inactivated at 100°C for 15 min. Only those samples that were repeatedly positive for HCV RNA were presented. In parallel, the cytotoxic activity of these infected cells was also assayed.

RESULTS

HCV RNA profile of the patients
Table 1 shows the HCV RNA analysis done in the HCV-infected...
patients. All were positive for HCV RNA in serum samples. Genomic sequences of HCV were also identified in nine out of 11 PBMC samples. HCV genotype analysis showed a prevalence of 100% and 66% of genotype II (1b) in serum samples and PBMC, respectively. Co-infection with two genotypes was also observed in one serum sample (genotypes I and II) and two PBMC samples (genotypes I-IV and I-II). These results were confirmed by typing serum and PBMC samples with individual sets of primers for each genotype.

**Cell subpopulations in HCV-infected patients**

Figure 1 shows the percentage of circulating cell subpopulations (T, B lymphocytes, monocytes and NK cells) in healthy donors and HCV-infected patients assessed by flow cytometry. The percentages observed for the different cell subpopulations were comparable between patients and controls. NK cells accounted for 11.2% ± 3.4% and 13.8% ± 5.5% of the total cell population in controls and patients, respectively.

**Table 1. HCV RNA profile in HCV-infected patients**

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HCV RNA and virus genotypes were assessed as described in Patients and Methods using the Inchauspe et al. [13] protocol and the Okamoto et al. method [14], respectively.

**NK cells in HCV infection**

Figure 2 illustrates the NK spontaneous cytotoxic activity (using K562 cells as targets) in PBMC from HCV-infected patients in the presence or absence of IL-2. The basal NK cytotoxic activity in HCV-infected patients was significantly diminished in all E:T cell ratios \((P<0.001)\) when compared with non-stimulated controls. Treatment with 100 U/ml IL-2 for 18 h increased significantly \((P<0.01)\) the NK activity in both patients and controls. However, upon IL-2 stimulation, NK activity in HCV-infected patients remained significantly lower \((P<0.01)\) than that of controls at all E:T cell ratios.

In order to determine if the decreased NK cytotoxicity against K562 cells was due to the presence of suppressor cells, PBMC from patients were depleted from monocytes and B cells by incubation on plastic dishes and passage through nylon wool columns. The depletion of adherent and B cells did not increase NK spontaneous cytotoxicity. The percentage of cytotoxicity observed in PBMC from HCV-infected patients \((9.1 \pm 1.8\%, 5.7 \pm 1.5\%)\) and controls \((19.6 \pm 4.0\%, 40.6 \pm 9.7\%)\) was comparable to the lymphocyte-enriched cell population \((10.6 \pm 5.8\% \text{ and } 7 \pm 3.7\%)\), respectively. Addition of IL-2 to PBMC and to lymphocyte-enriched cell populations increased NK cytotoxic activity in HCV-infected patients in a comparable fashion \((28.7 \pm 7.1 \text{ versus } 40.3 \pm 9.5 \text{ and } 20.4 \pm 10.3 \text{ versus } 25.1 \pm 10.4, \text{ E:T ratios } 40:1 \text{ and } 20:1, \text{ respectively, } n = 7)\).

Although IL-2 partially re-established NK cytotoxicity, this enhancement was far less than the values observed in normal PBMC stimulated with IL-2 \((69.8 \pm 10.7 \text{ and } 54.5 \pm 10.6 \text{ for E:T ratios of } 40:1 \text{ and } 20:1, \text{ respectively, } n = 7).\)

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Figure 3 represents LAK activity of PBMC against U937 cells from controls and HCV-infected patients. No differences were observed between both groups. A similar response was observed using K562 cells as targets (results not shown).

**NK and LAK activity in HCV-infected patients**

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Figure 3 represents LAK activity of PBMC against U937 cells from controls and HCV-infected patients. No differences were observed between both groups. A similar response was observed using K562 cells as targets (results not shown).

incubated with the specified serum samples for short (4 h) or long (48 h) periods of time. At 4 h, no differences were observed when the various treatments were compared. However, at 48 h, in both donors, a marked decrease in NK cytotoxic activity was observed in three of the four samples incubated with HCV + serum. The other serum samples did not affect NK spontaneous cytotoxicity.

NK cytotoxic activity of in vitro infected PBMC with a HCV RNA-positive serum

In order to investigate in more detail the effect of HCV on NK cytotoxicity, in vitro cell infection experiments were performed using PBMC from uninfected controls incubated with a HCV RNA-positive serum. Viral RNA sequences could be identified in three out of 10 donors. A typical result of a successful infection experiment is represented in Fig. 4. The Fig. 4a illustrates the results of nested PCR analysis done in one PBMC donor cultured with different dilutions of a HCV RNA-positive serum. After 48 h of cell culture, genomic sequences of HCV were detected in the PBMC (columns ii–iv). No viral RNA was identified in the last cell wash (column v), suggesting that virus detection was associated with infection of these cells and not with viral particles present in the culture. Heat-inactivated HCV RNA serum did not infect PBMC (column vi). Furthermore, by using a strand-specific reverse transcriptase (RT)-PCR assay in which the sense primer was used for cDNA synthesis, we could identify negative strands of HCV RNA in this PBMC sample (Fig. 4b). Treatment of these cells with trypsin and RNase before RNA isolation and PCR analysis did not modify the above results, suggesting that detection of HCV RNA was not attributable to viral adherence to the cells.

NK cytotoxic activity was assayed in the three in vitro infected PBMC donors. Significantly diminished activity was observed in these infected cells compared with uninfected controls ($P < 0.05$) (Fig. 5). This effect was dependent on the concentration of serum used, since inhibition of NK activity increased with serum concentration, reaching total inhibition with undiluted serum. In addition, a cytotoxicity assay in which these in vitro infected cells (with undiluted serum) were used as target against autologous NK cells was performed. Specific $^{51}$Cr release was observed in the experiments performed with the three different donors (6.11 ± 0.88%, 4.5 ± 1.6%, E:T ratios 5:1 and 2:5:1, respectively).

**DISCUSSION**

NK cells represent an important lymphocyte population involved in the immune response. Both cytotoxic and immunoregulatory functions of these cells have been described [10,11]. However, the role of NK cells in human disease is poorly understood. Current evidence seems to indicate that a decreased NK cell number or cytotoxic activity is often associated with the development and/or progression of cancer, acute or chronic viral infection, autoimmune diseases, immune deficiency syndromes and psychiatric illness [10]. The anti-viral activity of NK cells has been well studied in animal models [10,11]. In humans, there have been few reports that show a positive correlation between sensitivity to viral infection and depressed NK activity [10,11,16].

HCV infection is associated with a high percentage of chronic hepatitis [1,2]. The mechanisms responsible for this clinical outcome are at present unknown. Several hypotheses have been postulated to explain possible pathways leading to chronic liver damage, and these include: (i) escape from immunosurveillance;
(ii) suppression of host effector responses; and (iii) generation of viral variants [17]. A tentative assumption is that an altered immune response is partially responsible for viral chronicity [3–5]. Evidence to support this hypothesis may be linked to the fact that IFN-α therapy is effective in 40–50% of patients with chronic hepatitis [18], HCV-infected patients may develop autoimmune hepatitis [19], the histopathologic lesions observed in HCV-infected patients resemble those observed in HBV infection [20], and patients’ PBMC may be infected by the virus [21–24]. However, these effects may not be related to T lymphocyte hyper- or hyporesponsiveness. Previous reports demonstrated that T lymphocytes from HCV-infected patients proliferate in response to specific antigen or under stimulation with anti-CD3 and anti-CD28 MoAbs [3,7,9]. Likewise, cytotoxic T lymphocyte (CTL) response does not seem to be altered in HCV infection [6,8]. The effect of viral infection on the functional roles of other lymphocyte populations has not been fully assessed.

In the present study, we evaluated the NK cytotoxic activity of HCV-infected patients and the effect of IL-2 in modulating such activity. Cytotoxic assays were carried out in both sporadic and post-transfusion non-treated HCV-infected patients. The results show that basal NK cytotoxic activity is significantly diminished in HCV-infected patients, regardless of the fact that PBMC or NK-enriched cell populations were used in the assay. This diminished NK activity does not seem to be related to a deficit in the circulating NK cells, since the percentage of this cell population in HCV-infected patients was comparable to that observed in healthy controls. Moreover, no major absolute differences in other lymphocyte populations were observed between patients and controls.

As shown in Table 1, nine (82%) of the 11 PBMC samples studied were positive for HCV RNA, with a prevalence of genotype II (1b). Despite the fact that two PBMC samples were negative for HCV RNA, the cytotoxic activity observed in these samples was comparable to the infected ones. HCV-infected patients may be attributed to the effect of suppressive cells and/or factors present in PBMC-infected cells. To address this aspect, basal NK cytotoxic activity in HCV-infected patients was assayed using PBMC depleted of monocytes and B cells and purified NK cells. The results showed that, in spite of the depletion of monocytes and B cells, NK cytotoxic activity was still significantly low compared with healthy controls. Therefore, monocytes and B cells or their

soluble factors do not seem to be implicated in the diminished NK activity observed in HCV-infected patients.

We also assessed the effect of IL-2 on NK cell cytotoxicity. Even though IL-2 increases NK cytotoxic activity [10], a short priming with IL-2 was not sufficient to overcome completely the impaired cytotoxic response of cultured human NK cells from HCV-infected patients. Thus, IL-2 was only partially able to restore NK cytotoxic activity in these patients, this activity being significantly lower than that observed in PBMC from healthy controls, regardless of cell culture conditions. Furthermore, it is important to add that LAK activity in HCV-infected patients was comparable to that of controls.

Several authors have reported infection of PBMC and human cell lines by HCV [21–26]. Following the same approach, we incubated PBMC from healthy controls for 48 h with a HCV RNA-positive serum, confirmed virus infection using RT-PCR and evaluated its effect on NK cytotoxic activity. Presence of positive or negative strands of HCV RNA was confirmed by nested PCR in these cells, suggesting that viral infection was achieved. The NK activity of the infected cells was impaired compared with PBMC incubated with NHS. Moreover, the results obtained in cytotoxicity assays using autologous infected cells suggest that non-infected NK cells are able to lyse infected PBMC.

Diminished NK cytotoxicity seems to be specific to HCV infection, since the spontaneous cytotoxic activity of PBMC incubated with serum from other viral hepatitis infections (HAV, HBV) or autoimmune hepatitis was comparable to that of PBMC incubated with NHS. Overall, the above findings support the data obtained with PBMC from HCV-infected patients and suggest a direct correlation between HCV infection and a deficient NK spontaneous cytotoxic response.

Impairment of NK cytotoxic activity has also been reported in other models of viral infection, including Epstein–Barr virus, HIV and measles virus [10]. In this regard, our results are in agreement with the findings of Casali et al. [27], who reported a failure in the NK activity of measles virus-infected lymphocytes after 48 h of virus infection. The mechanism by which these viruses disturb the specific immune function are still unknown, but direct modulation by viral proteins and/or lymphocyte effector signals upon viral infection may represent two major possibilities.

The involvement of some cell receptors, like MHC class I molecule receptors, as a negative feedback for NK cytotoxicity activity represent another interesting model that could explain the depressed NK activity observed in HCV-infected patients [28]. Viral proteins may interact directly or indirectly with MCH class I inhibitory receptors present at the surface of NK cells, generating an inhibitory signal for the cytotoxic response.

In conclusion, we have demonstrated that NK cytotoxic function is impaired in HCV-infected patients regardless of NK circulating numbers and LAK activity. Further insights are needed to understand a possible link to chronic liver disease induced by HCV.

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