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In vitro effect of amantadine and interferon α-2a on hepatitis C virus markers in cultured peripheral blood mononuclear cells from hepatitis C virus-infected patients

Julio Martín [a,b], Sonia Navas [a,b], Mario Fernández [a,b], Miguel Rico [a,b], Margarita Pardo [a,b], Juan Antonio Quiroga [a,b], Friederike Zahm [c], Vicente Carreño [a,b,*]

[a] *Department of Hepatology, Fundación Jiménez Díaz, Avda. Reyes Católicos, 2, 28040 Madrid, Spain*
[b] *Fundación para el Estudio de las Hepatitis Virales, Madrid, Spain*
[c] *F. Hoffmann-La Roche, CH-4070, Basel, Switzerland*

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Abstract

The effects of amantadine (1–5 μM) and interferon α (IFNα)-2a alone (1000 IU/ml) and combined, have been studied in cultured peripheral blood mononuclear cells (PBMC) from 15 chronic hepatitis C patients and ten healthy donors. Amantadine itself did not affect cell viability and had minor effects on the response to mitogens by PBMC. Four patients (27%), but no donors, had hepatitis C virus (HCV) core and NS3-specific proliferative responses. Amantadine suppressed these responses in all cases and its antiproliferative effect was greater than that of IFNα (Mann–Whitney's *U*-test: $P < 0.05$ in both cases). All PBMC cultures from patients, but none from donors, were HCV RNA positive. Amantadine alone or combined with IFNα dose-dependently reduced HCV RNA content in individual PBMC (Wilcoxon's signed rank test: 1 μM, $P < 0.05$; 2 μM, $P < 0.02$; and 5 μM, $P = 0.16$) with respect to untreated cultures. In addition, 7, 13 and 20% of PBMC cultures became HCV RNA negative with 2 μM amantadine alone, IFNα alone and their combination, respectively. Finally, in contrast to IFNα, amantadine did not modify expression of 2′,5′-oligoadenylate synthetase activity or the spontaneous or mitogen-stimulated IFNγ and interleukin 10 production. In conclusion, these effects in PBMC from HCV patients suggest that the amantadine/IFNα combination might be considered a therapeutic option for treating chronic hepatitis C patients.

Keywords: Amantadine; Interferon α; Hepatitis C virus; PBMC

* Corresponding author. Tel.: + 34-91-543-19-64; fax.: + 34-91-544-92-28.
E-mail address: vcarreno@uni.fjd.es (V. Carreño)

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1. Introduction

The hepatitis C virus (HCV) is 9.7 kb in length, single stranded (ss) RNA virus of plus polarity, that is closely related to flavi- and pestiviruses (Van der Poel et al., 1994; Kolykhalov et al., 1997). Genetic heterogeneity has been reported among different HCV isolates; accordingly, these have been classified into several genotypes and subtypes (Simmonds, 1995). Diverse studies have shown that HCV infects peripheral blood mononuclear cells (PBMC) from HCV-infected patients (Zignego et al., 1992; Müeller et al., 1993; Navas et al., 1994; Lerat et al., 1996; Navas et al., 1998), and cultures of PBMC derived from chronic hepatitis C patients have recently been used to study HCV-host interaction (Martín et al., 1996, 1998b). Chronic hepatitis C is associated with progressive liver disease (Di Bisceglie et al., 1991; Tsukuma et al., 1993) and hepatocellular damage caused by HCV may be immune-mediated (Koziel et al., 1992; Botarelli et al., 1993; Minutello et al., 1993; Ferrari et al., 1994; Hoffmann et al., 1995).

Interferon α (IFNα) and its combination with ribavirin are the only approved drug regimens for the treatment of chronic hepatitis C. IFNα induces normalization of liver function tests in about 50% of treated patients, although most of them relapse when therapy is stopped (Davis et al., 1989; Di Bisceglie et al., 1989; Pardo et al., 1995). For this reason, novel drugs are needed for the treatment of chronic hepatitis C. Amantadine (1-adamantanamine) is an antiviral agent with proven efficacy against influenza A virus; in addition, it has activity against rubella, paramyxo-, arena- and flaviviruses (Davies et al., 1964; Oxford and Schild, 1965; Kato and Eggers, 1969; Welsh et al., 1971; Pfau et al., 1972; Koff et al., 1980; Leibowitz and Reneker, 1993). Amantadine hydrochloride has recently been used for the treatment of chronic hepatitis C patients in which previous IFNα therapy had failed, with promising results since it induced normalization of aminotransferase levels and disappearance of serum HCV RNA in 27% of the patients (Smith, 1997). For these reasons, the aim of this study was to investigate in vitro the effects of amantadine alone and in combination with recombinant human IFNα on cultured PBMC isolated from chronic hepatitis C patients.

2. Materials and methods

2.1. Patients

Fifteen consecutive anti-HCV Ab positive (determined by INNOTEST HCV Ab III™, and confirmed by INNO-LIA HCV Ab III™; Innogenetics NV, Zwijnaarde, Belgium) patients with histologically proven chronic HCV infection (Desmet et al., 1994), without cirrhosis, were selected. They were positive for HCV RNA of subtype 1b in serum and PBMC, as detected by the method described below. Other causes of liver disease such as hepatitis B virus or hepatitis G virus infection (Madejón et al., 1997), cytomegalovirus or Epstein–Barr virus, and infection by human immunodeficiency virus as well as non-viral causes (autoimmunity, metabolic, etc.) were excluded. None of the patients had received previous antiviral or immunomodulatory therapy. As controls, ten age-matched healthy blood donors were included. This study was conducted in accordance with the Declaration of Helsinki on human experimentation.

2.2. Cell cultures

PBMC from patients and controls were isolated by Ficoll-Hypaque gradient sedimentation (SEROMED™, Biochrom KG, Berlin, Germany) from fresh, heparinized, venous blood, washed twice with phosphate-buffered saline (PBS), and suspended in RPMI 1640 (Serva Feinbiochemica GmbH, Heidelberg, Germany), supplemented with 10% heat-inactivated fetal bovine serum (Imperial Laboratories, Andover, UK), 20 mM Hepes, 2 mM glutamine and antibiotics. The cell viability was assessed by the trypan blue exclusion test. PBMC were seeded at 2×10^6 viable cells/ml in 12-well tissue culture clusters (Costar, Cambridge, MA), at 37°C in a humidified atmosphere containing 5% CO_2, and cultures were maintained in duplicate for 7 days with medium alone (un-

stimulated cultures) or stimulated with 10 μg/ml phytohemagglutinin (PHA) (Sigma, St. Louis, MO) plus 10 μg/ml *Escherichia coli* lipopolysaccharide (LPS) (Sigma). After 16 h of mitogen stimulation, these duplicate cultures were left without treatment or treated with amantadine hydrochloride (kindly provided by Instituto Llorente, Madrid, Spain) alone, recombinant human IFNα-2a (kindly provided by F. Hoffmann-La Roche AG, Basel, Switzerland) alone, or with the amantadine/IFNα-2a combination. The range of concentrations of amantadine (from 1 to 5 μM) and the dose of IFNα (1000 IU/ml) were selected so that these included the clinically relevant serum concentrations achieved in vivo, and were equivalent to previously reported in vitro effective doses (Davies et al., 1964; Tominack and Hayden, 1987; Douglas, 1990; Lanford et al., 1994; Shimizu and Yoshikura, 1994). At the end of the incubation period, supernatants were collected, centrifuged, filtered, aliquoted and stored at −80°C; adherent PBMC were harvested and pooled with cells obtained in the centrifugation of supernatants. Proliferation under each condition was assessed using a nonradioactive cell proliferation assay (CellTiter 96, Promega, Madison, WI). The possible drug-related cytotoxicity was also tested at the end of the incubation period by determination of lactate dehydrogenase release into culture supernatants (Martín et al., 1998b).

PBMC from patients and controls (1×10^5 viable cells/100 μl) were also seeded in 96-well tissue culture clusters (Costar) and maintained in triplicate for 7 days with medium alone or stimulated with 1 μg/ml recombinant core or NS3 HCV antigens (Mikrogen GmbH, Munich, Germany), either in the absence and in the presence of amantadine (2 μM), IFNα-2a (1000 IU/ml), or the amantadine/IFNα-2a combination at the same doses. Due to limitations in the PBMC samples, the amantadine doses of 1 and 5 μM were not tested in the proliferation assay. The content of HCV core and NS3 proteins has previously been used to test the HCV antigen-specific PBMC proliferation (Diepolder et al., 1995; Lechmann et al., 1996). HCV antigen-specific PBMC proliferation was assessed as above and results were expressed as the average of stimulation index (SI) with respect to the proliferation observed in cultures with medium alone. A SI equal to or greater than 3 times the proliferative response observed in PBMC cultured in medium alone, in triplicate cultures in the presence or absence of 1 μg/ml of each HCV recombinant antigens, was considered significant.

2.3. HCV RNA detection

After RNA extraction from serum and PBMC samples, reverse transcription and nested-polymerase chain reaction (RT-PCR) were performed using universal primers from the 5′ non-coding region of HCV genome (McOmish et al., 1994). Typing and subtyping of HCV genomes were performed in PBMC samples of all patients, using RT-PCR followed by restriction fragment length polymorphism analysis of RT-PCR products, as previously described (McOmish et al., 1994; Davidson et al., 1995; Navas et al., 1997). The specificity of HCV RNA detection was assessed as previously described (Navas et al., 1994). The final PBMC wash mixture was processed and included as a specificity control in the PCR analysis of PBMC. To avoid false positive results, standard prevention measures were followed (Kwok and Higuchi, 1989). Finally, the antiviral effect of experimental treatments was established by quantitating HCV RNA in cultured PBMC, using a modification of the serum standard protocol of Amplicor™ HCV Monitor assay (Roche Diagnostic System, Branchburg, NJ) (Martín et al., 1998a). Briefly, 1 μg of PBMC-derived total RNA was co-extracted and subjected to co-amplification with a synthetic RNA of known concentration used as internal control; after the detection step, the results were calculated using a formula modified so as to give the HCV RNA content on a per μg total RNA basis.

2.4. Testing of 2′,5′-oligoadenylate (2-5A) synthetase activity

A portion of the cells obtained from each patient or control was washed with PBS and treated with lysis buffer (0.5% [vol/vol] NP-40, 25 mM Hepes, 5 mM $MgCl_2$, 10% [vol/vol] glycerol) con-

taining a mixture of protease inhibitors (1 mM PMSF, 10 μM leupeptin, 1 mM benzamidine and 1 mM EDTA). The activity of the enzyme 2-5A synthetase was measured in cell lysates by a commercial RIA (Eiken Chemical, Tokyo, Japan) and the results were expressed as the amount of enzyme per 50 000 viable cells.

2.5. Cytokine secretion into culture supernatants

Levels of IFNγ, interleukin (IL)-2 and IL-10 were measured in duplicate using commercially available ELISA tests (Medgenix Diagnostics, Fleurus, Belgium). To detect specifically the heterodimeric IL-12, an ELISA test was used as described previously (D'Andrea et al., 1992; Zhang et al., 1994; Quiroga et al., 1998) with reagents kindly provided by Dr M.K. Gately (Hoffmann-La Roche, Nutley, NJ).

2.6. Statistical analysis

For the comparison of means and frequencies the Student's *t*-test and Fisher's exact test were used, respectively; in addition, data were analyzed by the non-parametric Wilcoxon's matched-pairs signed rank test (for paired samples) and Mann–Whitney's *U*-test (for comparisons between two independent groups).

3. Results

3.1. Nonspecific PBMC proliferation

Amantadine in the physiological range (1–5 μM) did not affect cell viability in unstimulated cultures (data not shown) and had minor effects on the proliferative response to mitogens of PBMC isolated from HCV patients (untreated: 100%; 1 μM [mean ± standard error of the mean]: 115.0 ± 5.7%; 2 μM: 112.4 ± 6.4%; and 5 μM: 112.6 ± 7.8%) and healthy donors (untreated: 100%; 1 μM: 95.6 ± 7.1%; 2 μM: 104.3 ± 9.3%; and 5 μM: 93.8 ± 11.3%). Higher doses of amantadine (50 and 500 μM) were only investigated in PBMC from donors; the dose of 50 μM did not affect PBMC proliferation (96.9 ± 6.0%), whereas the dose of 500 μM showed a marked anti-proliferative effect (46.0 ± 8.0%; Student's *t*-test: $P < 0.001$). Mitogen-stimulated PBMC proliferation was also decreased by treatment with 1000 IU/ml IFNα-2a alone (HCV patients: 77.2 ± 5.7%, and healthy donors: 74.2 ± 5.7%; Student's t-test: $P < 0.01$ in both cases) and, to a similar extent as with IFNα alone, with the combination of amantadine plus 1000 IU/ml IFNα-2a (HCV patients, amantadine: 1 μM: 76.1 ± 6.1%; 2 μM: 59.8 ± 4.7%; and 5 μM: 67.6 ± 4.9%; healthy donors, amantadine: 1 μM: 73.1 ± 5.7%; 2 μM: 68.6 ± 7.6%; and 5 μM: 74.0 ± 6.3%; $P < 0.01$ in all cases with respect to the corresponding dose of amantadine alone). No differences were observed between the amount of lactate dehydrogenase released in mitogen-stimulated cultures without treatment, and those treated with amantadine alone, IFNα alone or the amantadine/IFNα combination (data not shown) excluding the possibility of drug-related cytotoxicity involving cytolysis.

3.2. HCV antigen-specific PBMC proliferation

Four of the fifteen chronic HCV-infected patients (27%), but none of the healthy donors, had HCV antigen-specific PBMC proliferative response (Fisher's exact test: $P = 0.11$), with SI equal to or greater than 3 times the proliferative response observed in cultures with medium alone. In addition, the PBMC from these four patients concurrently responded to both core (range of SI in these four patients: 4–8) and NS3 (range of SI: 3.5–7) antigens (Table 1). Amantadine alone (2 μM) abolished proliferative responses in these four cases (SI: 1–1.4 in both antigens). IFNα-2a alone (1000 IU/ml) also suppressed the HCV antigen-specific responses in such cases but to a lesser extent than amantadine alone, as shown by the comparison of the SI values in only the four cases with proliferative responses (SI: 1.6–2.7 and 1.5–2.4 for core and NS3, respectively; Mann–Whitney's *U*-test: $P < 0.05$ in both cases). Finally, in a similar way to amantadine alone, the amantadine/IFNα-2a combination completely suppressed the PBMC proliferative responses in the four cases (SI: 1–1.4 in both antigens) and this suppression was also significantly greater than that induced by

IFNα-2a alone in the same four cases (Mann–Whitney's U-test: $P < 0.05$ in both antigens). Previous experiments using tetanus toxoid as control antigen in a limited number of cultures showed that these effects were not restricted to HCV antigens (data not provided).

3.3. Antiviral effect of experimental treatments

All PBMC cultures from HCV patients, but none from donors, were HCV RNA positive by RT-PCR with or without mitogens (Fisher's exact test: $P < 0.05$). Furthermore, none of the final PBMC washes gave a positive result, confirming that HCV RNA positivity in PBMC was not due to residual serum contamination. Regarding the viral concentration in mitogen-stimulated PBMC (Table 2), the dose of 2 μM amantadine reduced the amount of HCV RNA (number of copies/μg total RNA) by 74%, while the HCV RNA concentration was only reduced by 57 and 69% with 1000 IU/ml IFNα-2a alone and the combination of 2 μM amantadine plus 1000 IU/ml IFNα-2a, respectively (Table 2). The differences in HCV RNA content between untreated PBMC and cultures treated with 1 or 2 μM amantadine were statistically significant (Wilcoxon's matched-pairs signed rank test: $P < 0.05$ and $P < 0.02$, respectively).

For individual PBMC samples, different degrees of reduction in HCV RNA concentration in mitogen-stimulated PBMC were obtained after treatment with 1, 2 and 5 μM amantadine alone and in combination with 1000 IU/ml IFNα-2a (Table 2). HCV RNA became negative in up to 3/15 (20%) PBMC cultures from HCV patients (not significant as compared to untreated cultures: Fisher's exact test, $P = 0.11$; Table 2). However, the effects of amantadine alone on HCV RNA in PBMC were found to be dose-related and statistically significant (Fisher's exact test: 1 μM: 3/15, $P = 0.11$; 2 μM: 5/15, $P < 0.05$; and 5 μM: 6/15, $P < 0.01$) based on the disappearance of (or > 75% reduction in) HCV RNA content (Table 2).

The three cases in which HCV RNA disappeared in PBMC when treated with the amantadine/IFNα-2a combination had lower basal HCV RNA concentration than the rest of the cases (median: 3 copies/μg RNA, range: 2–15 copies/μg RNA; vs median: 14 copies/μg RNA, range: 4–329 copies/μg RNA, respectively) although the difference did not reach statistical significance (Mann–Whitney's U-test: $P = 0.08$).

3.4. Induction of 2-5A synthetase activity

Basal levels of 2-5A synthetase activity in lysates of PBMC from healthy controls was significantly higher than in HCV patients (Mann–Whitney's U-test: $P < 0.05$; Fig. 1). IFNα-2a significantly increased 2-5A synthetase activity in PBMC cultures from HCV patients and healthy

Table 1
Proliferative response of peripheral blood mononuclear cells (PBMC) from hepatitis C virus (HCV) patients against recombinant HCV core and NS3 antigens

Amantadine (μM)	IFNα-2a (IU/ml)	Proliferation with HCV core[a]		Proliferation with HCV NS3[a]	
		Yes (SI)	No (SI)	Yes (SI)	No (SI)
0	0	4/15 (4–8)	11/15 (1–2)	4/15 (3.5–7)	11/15 (1–2)
2	0	0/15	15/15 (1–1.5)[b]	0/15	15/15 (1–1.5)[b]
0	1000	0/15	15/15 (1–2.7)	0/15	15/15 (1–2.5)
2	1000	0/15	15/15 (1–1.5)[b]	0/15	15/15 (1–1.5)[b]

[a] The 'yes/no' columns indicate the presence or absence, respectively, of a proliferative response with stimulation index (SI) equal to or greater than 3 times the proliferative response observed in PBMC cultured in medium alone. The range of SI values is between parentheses.

[b] Considering only the values obtained in the four patients with HCV antigen-specific PBMC proliferative response, their SI values were significantly lower in PBMC cultures treated with amantadine alone or the amantadine/IFNα-2a combination than in those treated with IFNα-2a alone (Mann–Whitney's U-test: $P < 0.05$ in both cases).

Table 2
Number of cases with reduction or disappearance of hepatitis C virus (HCV) RNA, and mean HCV RNA concentration, in cultured mitogen-stimulated peripheral blood mononuclear cells (PBMC) from 15 HCV patients after in vitro experimental treatments

Amantadine (μM)	IFNα-2a (IU/ml)	Reduction in HCV RNA concentration[a]					HCV RNA concentration in copies/μg total RNA	Statistical analysis[d]
		>25%	>50%	>75%	Negative[b]	Negative or > 75% reduction[c]	(mean ± S.E.M.)	
0	0				0/15 (0%)	0/15	36.5 ± 21.4	
1	0	3/15 (20%)	2/15 (13%)	3/15 (20%)	0/15 (0%)	3/15 ($P = 0.11$)	15.1 ± 5.9	$p < 0.05$
2	0	5/15 (33%)	2/15 (13%)	4/15 (27%)	1/15 (7%)	5/15 ($P < 0.05$)	9.5 ± 3.1	$p < 0.02$
5	0	2/15 (13%)	2/15 (13%)	3/15 (20%)	3/15 (20%)[e]	6/15 ($P < 0.01$)	13.3 ± 6.6	n.s.
0	1000	0/15 (0%)	2/15 (13%)	3/15 (20%)	2/15 (13%)	5/15 ($P < 0.05$)	15.7 ± 6.8	n.s.
1	1000	3/15 (20%)	3/15 (20%)	4/15 (27%)	0/15 (0%)	4/15 ($P < 0.05$)	10.2 ± 2.1	n.s.
2	1000	3/15 (20%)	1/15 (7%)	3/15 (20%)	3/15 (20%)[e]	6/15 ($P < 0.01$)	11.4 ± 3.9	n.s.
5	1000	0/15 (0%)	0/15 (0%)	2/15 (13%)	3/15 (20%)[e]	5/15 ($P < 0.05$)	19.5 ± 9.6	n.s.

[a] Refers to the percentage of reduction in HCV RNA concentration in mitogen-stimulated PBMC samples treated in vitro with respect to the corresponding mitogen-stimulated, untreated PBMC cultures (all of them were positive).

[b] A negative result corresponds to a 100% reduction in HCV RNA content with respect to the mitogen-stimulated, untreated PBMC culture.

[c] Number of cases with disappearance or >75% reduction of HCV RNA content compared with untreated cultures (P values obtained by Fisher's exact test).

[d] Comparison between HCV RNA content in untreated and treated PBMC cultures using the Wilcoxon's matched-pairs signed rank test (n.s., not significant).

[e] Not significant as compared to untreated cultures (Fisher's exact test: $P = 0.11$).

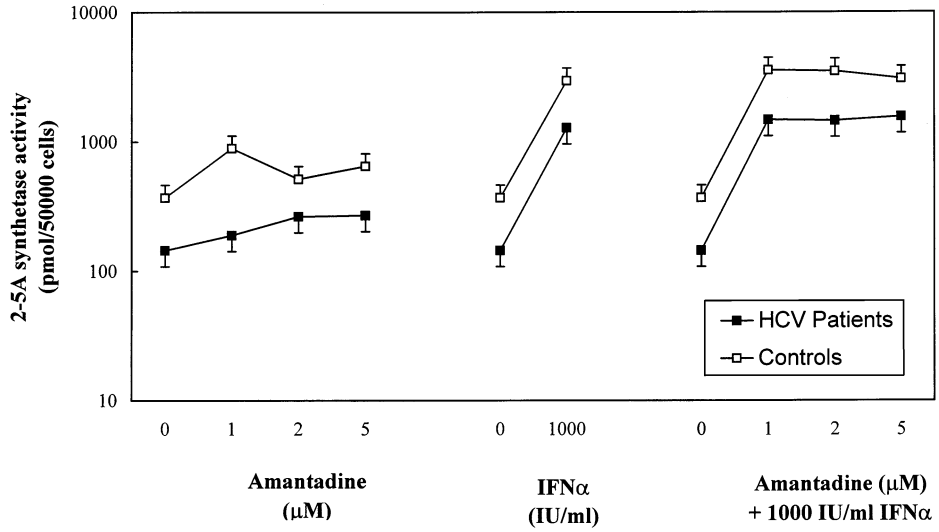

Fig. 1. Activity of 2′,5′-oligoadenylate (2-5A) synthetase in lysates of peripheral blood mononuclear cells (PBMC). After 7 days of mitogen-stimulated culture of 2×10^6 viable PBMC/ml from hepatitis C virus (HCV) patients (■) and healthy controls (□), an aliquot of the cells was lysed in the presence of protease inhibitors. Activity of 2-5A synthetase was measured by a sensitive RIA and results were expressed as the mean amount of enzyme per 50 000 viable cells ± S.E.M.

donors (Wilcoxon's matched-pairs signed rank test: $P < 0.01$ in both cases; Fig. 1). In contrast, treatment with amantadine alone had no major effect on this IFN-inducible activity, and no differences in this activity in PBMC were observed between treatments with IFNα-2a alone and with the amantadine/IFNα-2a combination in HCV patients or controls (Fig. 1).

3.5. Cytokine secretion into culture supernatants

Production of IL-2 and IL-12 was very low or undetectable in most PBMC cultures from HCV patients and healthy donors, either with or without mitogens. In addition, the treatment with amantadine alone, IFNα-2a alone, or the amantadine/IFNα-2a combination did not affect IL-2 and IL-12 production (data not shown). Spontaneous IFNγ and IL-10 production by PBMC from HCV patients and donors was similar (data not shown). However, under mitogenic stimulation, IFNγ production by PBMC from HCV patients was significantly greater than by PBMC from donors (Mann–Whitney's U-test: $P < 0.02$), while IL-10 production was also higher in HCV patients than in donors but without statistical significance (Mann–Whitney's U-test: $P = 0.18$; Fig. 2). Amantadine did not change IFNγ or IL-10 production in nonstimulated cells from HCV patients and donors (data not shown) and did not modify their production in response to mitogens (Fig. 2). In contrast, IFNα-2a significantly increased mitogen-stimulated IFNγ and IL-10 production by PBMC from HCV patients (Wilcoxon's matched-pairs signed rank test: $P < 0.001$ in both cases) and healthy controls (Wilcoxon's matched-pairs signed rank test: $P < 0.02$ and $P < 0.05$, respectively), and differences between both groups remained significant in the case of IFNγ (Mann–Whitney's U-test: $P < 0.01$) and not significant in the case of IL-10 (Mann–Whitney's U-test: $P = 0.10$; Fig. 2). The effects of the amantadine/IFNα-2a combination on IFNγ and IL-10 production by PBMC in response to mitogens were similar to those observed with single IFNα-2a treatment (Fig. 2).

4. Discussion

Amantadine is an antiviral agent with proven efficacy against several viruses. Although its

mechanism of action is not well known, the drug appears to interfere in the early stages of viral replication with either the uncoating of the virus or the primary transcription of viral RNA (Skehel et al., 1977; Oxford and Galbraith, 1980; Hay et al., 1985; Skehel, 1992). In addition, amantadine has recently been shown to exert anti-HCV activity in chronic hepatitis C patients (Smith, 1997). For these reasons, it was aimed to investigate the effects of amantadine on PBMC from chronic hepatitis C patients, and the amantadine concentrations studied (1–5 μM) were in the range of the clinically relevant serum levels achieved during therapeutic administration of amantadine (Tominack and Hayden, 1987; Douglas, 1990).

As to its antiviral effect, amantadine alone decreased the mean amount of HCV RNA in PBMC, and a slightly less pronounced effect was observed with IFNα alone and the combination of both. In spite of the variability in the HCV RNA levels among individual patient cultures, a dose-dependent effect of amantadine alone in the reduction of HCV RNA content in individual PBMC was found. In addition, the 2 μM amantadine/IFNα-2a combination had better results both in reduction and in disappearance of HCV RNA in individual PBMC (up to 20% of cases), showing a greater effect than the same doses of amantadine and IFNα-2a alone. This dose of amantadine corresponds to the steady-state serum level achieved during therapeutic administration of 100 mg amantadine/12 h, which was the schedule used to test the in vivo efficacy of amantadine in chronic hepatitis C (Smith, 1997). The reason for the lack of an additive effect in the combination of 1 and 5 μM amantadine plus IFNα is unknown; however, a similar additive effect to that described above had been previously reported in the in vitro and in vivo inhibition of influenza A virus replication (Lavrov et al., 1968; Lukacsi et al., 1985; D'Agostini et al., 1996).

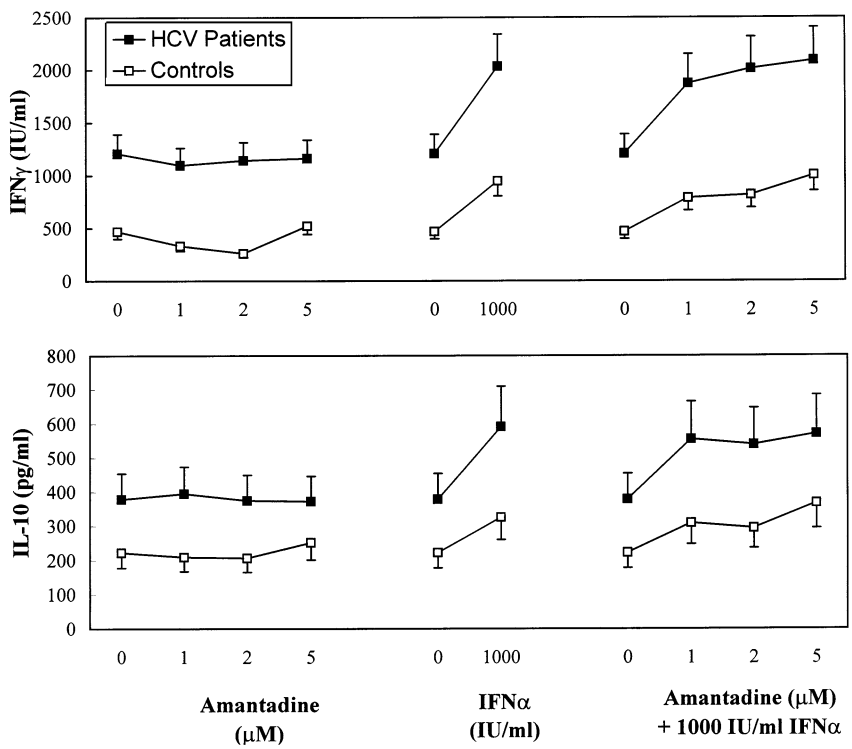

Fig. 2. Cytokine production into culture supernatants. Production of interferon (IFN) γ and interleukin (IL)-10 at the end of the 7-days culture period was measured in mitogen-stimulated peripheral blood mononuclear cells (PBMC) from hepatitis C virus (HCV) patients (■) and healthy controls (□) using commercial ELISA tests. Results were expressed as the mean amount ± S.E.M.

In agreement with the results obtained in vivo (Smith, 1997), disappearance of HCV RNA in PBMC seems to be related with a lower basal HCV RNA content. In addition, the antiviral effect of amantadine is not likely to be mediated by induction of IFN-inducible intracellular enzymes, since amantadine had no effects on 2-5A synthetase activity in PBMC.

Amantadine is a lysosomotropic compound that seems to be able to inhibit infection of viruses which depend on a low-pH step to enter the cytoplasm (Pérez and Carrasco, 1993; Castilla et al., 1994; Pérez and Carrasco, 1994). To date, it is unknown whether HCV enters into cells by direct fusion with the plasma membrane or by receptor-mediated endocytosis, requiring a low-pH step to initiate uncoating and to deliver RNA genome into the cytoplasm. In this sense, data supporting the hypothesis of a low-density lipoprotein receptor-mediated uptake of HCV in vitro, in cultures of several human hepatoma and porcine nonhepatoma cell lines, have recently been reported (Seipp et al., 1997). Thus, if HCV enters into cells by low-density lipoprotein receptor-mediated endocytosis and requires an endosomal low-pH, the amantadine-induced increase in endosomal pH would lead to inhibition of infection, as has been suggested for influenza A virus infection (Daniels et al., 1985; Marsh and Helenius, 1989). On the other hand, the sequestration of amantadine in the liver has been reported (Aoki and Sitar, 1988) with a liver to blood ratio of 19; this ability to achieve high liver concentrations along with its antiviral properties might indicate the way by which amantadine is effective in chronic hepatitis C patients. However, the precise mechanism(s) of amantadine effect in HCV infection (blocking the virus uptake, virus uncoating or early events in transcription) should be investigated in depth in the future.

At physiological concentrations (1–5 μM), amantadine did not affect mitogen-stimulated PBMC proliferation in HCV patients and healthy controls. However, amantadine (2 μM) suppressed HCV antigen-specific proliferative responses in PBMC from chronic hepatitis C patients (the other two doses of amantadine were not tested due to limitations in the PBMC samples), while IFNα also suppressed these responses but to a lesser extent. This antigen-specific proliferative response in the periphery is due to circulating HCV-specific T-cells (Botarelli et al., 1993; Ferrari et al., 1994; Hoffmann et al., 1995); perhaps, the amantadine effect might be related to a possible blocking of HCV antigen endocytosis by antigen-presenting cells, but the precise mechanism should be investigated. Since liver damage in chronic hepatitis C is likely caused by intrahepatic HCV-specific, activated T-cells, if the treatment with amantadine also suppresses the HCV antigen-specific proliferative response of intrahepatic T-cells, this fact might explain the improvement in biochemical markers in chronic hepatitis C patients treated with amantadine (Smith, 1997).

Several authors have reported that HCV patients have higher levels of certain cytokines in serum and their PBMC have increased production of several cytokines than healthy controls (Quiroga et al., 1994; Hagiwara et al., 1995; Cacciarelli et al., 1996; Cribier et al., 1998; Martín et al., 1998b, 1999). This fact suggests a baseline activation of T-cells and macrophages/monocytes in HCV patients. This situation remains unchanged in vitro since in the study amantadine did not modify cytokine production by PBMC from chronic HCV patients or healthy controls. By contrast, along with its antiviral effect and its suppression of HCV antigen-specific proliferative responses, IFNα also modulated cytokine production by PBMC. Finally, the amantadine/IFNα-2a combination suppressed antigen-specific proliferative responses in a higher degree than IFNα alone, and also modulated cytokine production in a similar way as IFNα alone. These data suggest that the combination of a chemotherapeutic agent—such as amantadine—with an antiviral/immunomodulatory drug—such as IFNα—might be beneficial for the treatment of chronic hepatitis C patients, but this should be proven in future studies.

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