International Immunopharmacology 91 (2021) 107302

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Preliminary report

Hepatitis delta: *In vitro* evaluation of cytotoxicity and cytokines involved in PEG-IFN therapy

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ARTICLE INFO

Keywords:
HBV
HDV
PEG-IFN
Cytokines

ABSTRACT

The treatment for hepatitis Delta virus (HDV) still consists of Pegylated interferon (PEG-IFN) combined with inhibitors of Hepatitis B virus (HBV) replication. In some patients may be occur a virological response, which means a negative HDV RNA 6 months after stopping treatment. In this study it was conducted an *in vitro* approach with the aim to mimic possible immunological events that are observed in patients responding to PEG-IFN therapy. Jurkat cells (human T lymphocyte cell line) were employed alone or co-cultured with THP-1 (human monocytic cell line) and stimulated with controls and HBV Surface Antigen (HBsAg), Small-Delta Antigen (SHDAg), and HBsAg + SHDAg combined. Twenty-four hours stimulation with SHDAg and/or HBSAg led to a toxic profile in a co-culture condition and cell supernatants were collected for cytokines quantification. PEG-IFN was added and cells were incubated for additional 24 h. Co-cultured cells incubated with the association (SHDAg + PEG-IFN) significantly produced levels of IFN-γ, IL-2 and IL-12. On the other hand, the HBsAg alone was able to inhibit the production of IFN-γ, suggesting that this antigen may hinder the treatment exclusively with PEG-IFN.

1. Introduction

First described by Rizzetto in 1977 [1], who carried out experiments with chimpanzees to demonstrate that a defective virus, dependent on the Hepatitis B virus (HBV), was the cause of the infection. Hepatitis Delta virus (HDV) classified as a Deltavirus genus, it is a hepatotropic, defective, single-stranded and negative polarity RNA virus. HDV shares the same surface protein antigen as the hepatitis B virus (HBsAg) to coat. Thus, the assembly of new virions is dependent on the simultaneous presence of HBV in infected cells [2]. Clinical practice reports of therapeutic success attributed to the prolonged use of recombinant PEGylated interferons (PEG-IFNs) can be found in the literature [3–5]. Virological response is usually more frequently achieved when PEG-IFN is co-administered with inhibitors of HBV replication [6–8]. Despite the considerable amount of information regarding the therapeutic use of different drug combinations, apparently, the virological response depends on the characteristics of the host's immune system, since the secretion of proinflammatory cytokines in patients responding to treatment seems to be a key point in fighting the infection [9,10].

The presence of certain cytokines in individuals responding to PEG-IFN treatment along with data suggesting that T-cell depletion may be one of the escape mechanisms of HDV [11] led us to hypothesize that

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https://doi.org/10.1016/j.intimp.2020.107302
Received 15 October 2020; Received in revised form 8 December 2020; Accepted 14 December 2020
Available online 1 January 2021

combination therapy might result in reducing the amounts of circulating HBsAg, and that this may be one of the mechanisms underlying therapeutic success. Our hypothesis is reinforced by the mechanism of cellular exhaustion produced by HBV, since the HDV envelope consists of HBsAg and thus, the immune response triggered by the presence of the virus could involve the same or related mechanisms [12].

Here, in this study, it was conducted an *in vitro* approach, by mimicking possible immunological events that are observed in patients responding to PEG-IFN treatment. An investigation was made on the influence of a microenvironment containing HBSAg and/or SHDAg in the secretion of cytokines by co-cultured cells. Moreover, the modulation of events involving the main inflammatory cytokines was assessed after the addition of PEG-IFN.

Preliminary experiments with Jurkat cells (ATCC TIB-152), a human T lymphocyte cell line, cultured with RPMI medium supplemented with 10% of Fetal Bovine Serum (FBS) and 0.1% of Gentamicin at 37 °C and 5% CO_2 atmosphere, were designed to standardize the concentrations of antigens used in each assay (data not shown). The following stimuli were used: 1) PBS - negative control; 2) LPS (L6529, Sigma) (500 ng/mL) - positive control; 3) HBsAg (Diasorin The Diagnostic Specialist - Salugge, Italy) (25 ng/mL); 4) SHDAg [13,14] (25 ng/mL) and 5) HBsAg + SHDAg (25 + 25 ng/mL (1:1)) and cytotoxicity were evaluated.

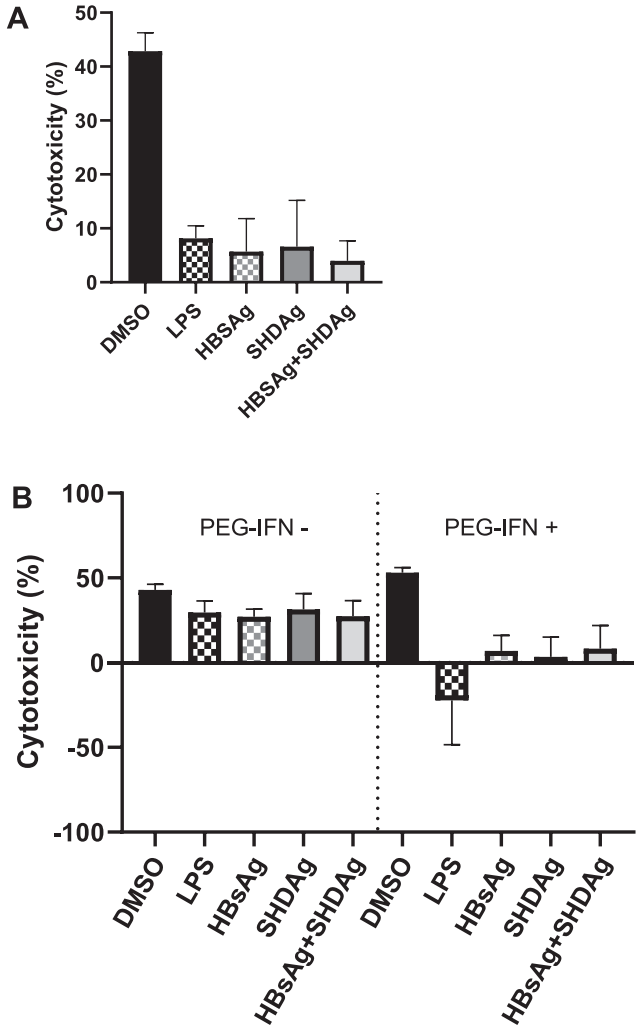

Fig. 1. Cytotoxicity (%) assay by MTT employing the following stimuli: LPS, HBsAg, SHDAg and SHDAg + HBsAg combined. Jurkat cells alone (A) and co-cultured (4:1 ratio) with THP-1 cells (B), before and after incubation with PEG-IFN (100 ng/mL). Results are expressed as mean ± S.D of triplicates from two independent experiments.

Fig. 1**A** showed that HBsAg and SHDAg, employed alone or combined in the Jurkat cell culture did not display any toxicity.

The next approach was carried out an assay employing a co-culture of lymphocytes (Jurkat cells) and monocytes (THP-1 cells) (ATCC TIB-202). Cells were co-cultured in a 4:1 ratio ($8x10^6$ cells/mL Jurkat and $2x10^6$ THP-1), with RPMI medium supplemented with 10% of Fetal Bovine Serum (FBS) and 0.1% of Gentamicin at 37 °C and 5% CO_2 atmosphere, mimicking the proportion of human peripheral blood mononuclear cells (PBMC) in the peripheral blood, where between 70 and 90% amount of the PBMC are lymphocytes and 10–20% are monocytes.

Interestingly, in the co-culture assay, employing THP-1 cells (Fig. 1**B**), all tested antigens were more cytotoxic. This phenomenon was not observed in the culture of Jurkat alone, when cells were stimulated by the same antigenic concentration (25 ng/mL) **(**Fig. 1**A)** [13].

Despite this observed cytotoxicity, we maintained the *in vitro* protocol with 4:1 co-culture of Jurkat and THP-1 cells, and 24 h of antigenic stimulation (25 ng/mL). After this period, a standardized concentration-dependent PEG-IFN solution (100 ng/mL; Pegasys, Roche) was added, as previously described by [12] and cells were further incubated for additional 24 h. After incubation, a cell viability assay was performed using tetrazolium-MTT assay (5 mg/mL, 10 μL each well) for 4 h. Cell viability values were expressed as a percentage relative to the absorbance (540 nm) determined in control cells that were treated with 10% (m/v) SDS solution. This assay was performed after 24 h incubation of cells under different stimuli and after additional 24 h incubation in the presence of PEG-IFN solution.

Viral antigens were able to induce cell death at levels comparable to those observed in the presence of LPS (positive control) (Fig. 1**B**). Noteworthy that, a decrease in the cytotoxicity following the addition of PEG-IFN could be remarked for all the stimuli tested.

The observation of HDV-related cytotoxicity was reported before [14], however, it is worth noting that cytotoxicity is more often evidenced in early cell culture periods, and this effect tends to decrease over time. Our experiments had a maximum duration of 48 h and our findings are in agreement with the authors reporting cell death in the initial periods of incubation. After standardizing the *in vitro* antigens stimulation and PEG-IFN incubation conditions, the next step was to investigate whether the cells in our assay were being stimulated to produce any cytokines. Quantification of IL-2, IL-12, and IFN-γ cytokines were performed using commercial ELISA assays (BD OptEIA™, BD Biosciences Franklin Lakes, NJ, USA), according to the manufacturer's instructions (Table 1). Regarding IL-2, it was observed that cells that did not undergo any antigenic stimulation nor PEG-IFN incubation (control) produced low levels of this cytokine, although some basal levels were still presented **(**Table 1**)**. When cells were stimulated with HBsAg, IL-2 secretion levels were found to be lower than those determined for the control, suggesting a possible inhibition of this pathway. Another observation was that IL-2 secretion in cells stimulated with SHDAg and later incubated with PEG-IFN increased significantly when compared to the control group ($p < 0.0001$), to cells incubated with LPS or HBsAg ($p < 0.0001$), and to cells stimulated with HBsAg + SHDAg ($p < 0.05$).

Regarding the production of IFN-γ, it only occurred when cells were co-cultivated in the presence of SHDAg followed by the incubation with PEG-IFN (Table 1**)**. Notably, cells that were stimulated with both antigens (HBsAg + SHDAg) did not secrete IFN-γ in a minimum detectable dose of 1.0 pg/mL, according to manufacture instructions, even after the addition of PEG-IFN. This result suggests a possible role of HBsAg as inhibitor of IFN-γ secretion. Production of IL-12 has been previously correlated with IL-2 production in HDV infected patients [9]. Similar to IL-2 and IFN-γ, the highest production of IL-12 in SHDAg-stimulated cells after the incubation with PEG-IFN was observed (Table 1**)**. The values obtained were statistically significant when compared to the negative control and LPS stimulated cells ($p < 0.001$), suggesting increased IFN-γ production after also increased IL-12 levels. Moreover, the incubation of SHDAg-stimulated cells with PEG-IFN was able to

Table 1
Quantification of IL-2, IFN-γ and IL-12 in the supernatant of cultured cells.

	Jurkat + THP-1 co-cultured antigen stimulated				
	Control	LPS	HBsAg	SHDAg	SHDAg + HBsAg
	pg/mL	pg/mL	pg/mL	pg/mL	pg/mL
Cytokine					
IL-2	2.24 ± 1.66	3.42 ± 2.09	ND	4.88 ± 4.58	5.18 ± 1.25
IFN-γ	ND	ND	ND	ND	ND
IL-12	82.5 ± 1.01[£]	76.78 ± 2.02[%]	75.35 ± 5.05[*]	75.00 ± 1.24[#]	122.14 ± 17.73[£,%,*,#]
	Jurkat + THP-1 co-cultured antigen stimulated with PEG-IFN				
	Control	LPS	HBsAg	SHDAg	SHDAg + HBsAg
	pg/mL	pg/mL	pg/mL	pg/mL	pg/mL
Cytokine					
IL-2	3.19 ± 2.10[a]	ND	ND	9.44 ± 4.75[a,b]	4.66 ± 2.90[b]
IFN-γ	ND	ND	ND	17.83 ± 1.18	ND
IL-12	84.45 ± 10.81[c,d]	87.98 ± 6.48[e,f]	88.57 ± 1.51 [g,h]	158.93 ± 56.59[c,e,g]	138.21 ± 5.58[d,f,h]

Levels (pg/mL) of IL-2, IFN-γ and IL-12 produced by cells stimulated with HBsAg and SHDAg, in the absence or presence of PEG-IFN (100 ng/mL). Results are expressed as mean ± S.D of triplicates from two independent experiments. ND = not detectable values. A two-way ANOVA statistical test was used, followed by Bonferroni post-test. In the presence of PEG-IFN, production of IL-2 was statistically significant in the analyses of the SHDAg-stimulated cells when compared to the negative control ($^{a}p < 0.0001$) and to HBsAg + SHDAg ($^{b}p < 0.05$) groups. Production of IFN-γ was only detected by SHDAg-stimulated cells in the presence of PEG-IFN treatment (17.83 ± 1.18). IL-12 was the only cytokine, without PEG-IFN treatment, that displayed a statistically significant production ($^{£, \%, *, \#}$ $p < 0.01$) after the SHDAg + HBsAg combined stimulus compared to the other groups. Similar to other cytokines, after PEG-IFN treatment, SHDAg alone and also combined with HBsAg stimulated cells to produce high levels of IL-12 compared to negative control, LPS and HBsAg ($(^{c,e,g}p < 0.001$) and ($^{d,f,h}p < 0.01$), respectively).

induce 2x more IL-12 production compared with those untreated cells.

The *in vitro* experiments described here indirectly suggest that the presence of HBsAg induces events leading cells to a kind of "exhaustion" profile. In the presence of this stimulus, IFN-γ production was undetected and, consequently, subsequent cellular events were impaired. Similar outcomes were previously reported by Schirdewahn et al (2017) in HDV infected patients. Furthermore, these data may explain why combination therapy with PEG-IFN and inhibitors of HBV replication has been shown to be more effective than PEG-IFN administration alone. While PEG-IFN seems to promote recovery of the IFN-γ pathway, inhibited in the presence of HDV, nucleoside analogues prevent a further depletion of the immune system by suppressing HBV replication and consequently diminishing the production of HBsAg.

In conclusion, our results suggest that the incubation of Jurkat + THP-1 cells with PEG-IFN was able to preserve cellular viability. Also, the addition of PEG-IFN in the co-culture could increase the production of important cytokines (IL-2, IFN-γ and IL-12) related to host immune response against the viral infection. Especially for SHDAg-stimulated cells, IFN-γ and IL-12 levels were restored and 2x more detected, respectively. In this context, other studies investigating cytokines produced by patients [9,10,15] also strongly suggest that chronic HDV infection leads to T cell exhaustion by an indirect effect caused by the concomitant presence of HBSAg, facilitating virus escape from the patient's immune response. Accordingly, combination therapy reduces the amount of circulating HBsAg, indirectly favoring effective cell recovery by IL-12 [15]. However, it is possible to infer that the balance leading to therapeutic success is closely linked to the variability of the immune system of each patient [16]. Further investigations of the main cytokine production pathways as well as other immunological parameters that may be unique in HDV infected patients are mandatory.

CRediT authorship contribution statement

Larissa Deadame Figueiredo Nicolete: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing, Visualization. **Celso Vladimiro Cunha:** Investigation, Resources, Writing - original draft, Writing - review & editing, Supervision, Project administration, Funding acquisition. **João Paulo tavanez:** Methodology, Validation, Formal analysis, Writing - review & editing. **Mariana Tomazini Pinto:** Methodology, Validation, Formal analysis, Writing - review & editing. **Evandra Strazza Rodrigues:** Methodology, Validation, Formal analysis, Writing - review & editing. **Simone Kashima:** Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition. **Dimas Tadeu Covas:** Resources, Writing - review & editing, Project administration, Funding acquisition. **Juan Miguel Villalobos-Salcedo:** Conceptualization, Resources, Writing - original draft, Writing - review & editing, Supervision, Project administration. **Roberto Nicolete:** Conceptualization, Investigation, Resources, Data curation, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Acknowledgments

FIOCRUZ (EncPapesVI-Fiocruz, grant no. 407540/2012-1) and CNPq (no. 470455/2013-6) provided support for the development of the study. We are thankful to FCT for funds to GHTM (UID/Multi/04413/20139).

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