THE EFFECT OF INTERLEUKIN-6 ON HEPATITIS B VIRUS REPLICATION IN PERIPHERAL BLOOD MONONUCLEAR CELLS *IN VITRO*

M. SIDORKIEWICZ[1*], B. JÓŹWIAK[1], Z. SULOWSKA[2], J. GREGER[1], U. LEWANDOWSKA[1]

[1]Department of Medical Biochemistry, Medical University of Lodz, 92-215 Lodz, Poland; [2]Microbiology and Virology Center, Polish Academy of Science, Lodz, Poland

Received January 19, 2004; accepted August 5, 2004

Summary. – Although the major target organ for hepatitis B virus (HBV) is the liver, the possibility of infection of peripheral blood mononuclear cells (PBMCs) with HBV has also been reported. This study was performed to analyze the course of HBV infection of PBMCs and to investigate the influence of interleukin-6 (IL-6) on the efficiency of infection of PBMCs with HBV *in vitro*. PBMCs isolated from a healthy donor were infected by exposing to a HBsAg-, HBeAg-positive serum in the presence or absence of exogenous IL-6. The efficiency of infection was estimated by HBV DNA determination in the cells and medium in the course of infection. The results of this study show that the presence of IL-6 during the PBMCs infection with HBV increased the efficiency of this infection.

Key words: Hepatitis B virus; peripheral blood mononuclear cells; interleukin-6; PCR

Introduction

HBV (the species *Hepatitis B virus*, the genus *Orthohepadnavirus*, the family *Hepadnaviridae*) is a small DNA virus, which replicates via a reverse transcription and similarly as other hepadnaviruses is considered to be highly hepatotropic (Robertson and Margolis, 2002). However, cells other than hepatocytes, including peripheral blood lymphocytes and monocytes, may also become infected with HBV (Roisman *et al.,* 1994). HBV has been reported to occur in PBMCs (Oesterreicher *et al.,* 1995). Moreover, some authors have used PBMCs to study HBV infection in a non-hepatic tissue (Cabrerizo *et al.,* 2002). The cell receptor-binding site for HBV was assigned to the preS (21-47) segment of the HBV envelope protein (Neurath *et al.,* 1986). However, the cell receptors for HBV have not been characterized until now (Paran *et al.,* 2001).

*E-mail: msidor@zdn.am.lodz.pl; fax: +4842-6782465.

Abbreviations: HBV = Hepatitis B virus; IL-6 = interleukin-6; PBMCs = peripheral blood mononuclear cells; PHA = phytohemagglutinin; p.i. = post infection

The possibility of HBV receptors presence was confirmed for human liver and hepatoma cells, B lymphocytes, monocytes and T cell lines activated by *Escherichia coli* lipopolysaccharide and concanavalin A (Neurath *et al.,* 1989; Petit *et al.,* 1991) . Thus, the adsorption of HBV to PBMCs seems to be a receptor-mediated process (Neurath *et al.,* 1990; Pontisso *et al.,* 1991). The question whether all further steps of HBV replication can be supported by human PBMCs remains open (Kock *et al.,* 1996; Stoll-Beckr *et al.,* 1997).

IL-6 is a pleiotropic cytokine that induces many biological activities including some aspects of the immune reaction and inflammatory responses (Heinrich *et al.,* 2003). In the liver, IL-6 regulates the synthesis of a broad spectrum of acute-phase proteins. IL-6 is also known to be a factor involved in the immunoregulatory perturbations in patients with chronic liver diseases. Serum levels of IL-6 are elevated in acute and chronic hepatitis B patients. Several studies have suggested that the IL-6 levels in serum of hepatitis B patients correlate with HBV replication and with the degree of liver damage (Tangkijvanich *et al.,* 2000).

Some searches have revealed that human IL-6 contains the recognition sites for the preS (21-47) segment of HBV

envelope protein, and that IL-6 and anti-IL-6 antibodies, respectively, inhibit the interaction of cells expressing a receptor for HBV with the preS (21-47) segment of the HBV envelope protein, encompassing the complementary attachment site for IL-6 (Neurath *et al.,* 1992) . The above finding led to a suggestion that IL-6 may mediate HBV-cell interactions (Heinz *et al.,* 2001).

To investigate the course of PBMCs infection with HBV and to estimate the influence of IL-6 on the efficiency of this infection we carried out an *in vitro* infection of PBMCs with a HBV-positive serum in the presence or absence of IL-6. We estimated the efficiency and time course of the infection by screening for HBV DNA in cultured PBMCs and growth media using a semi-quantitative PCR assay. We found that (i) the level of HBV DNA in PBMCs increased with the time and (ii) IL-6, when added to the cells with the virus, increased that level and consequently the efficiency of the infection of PBMCs with HBV *in vitro*.

Materials and Methods

HBsAg-, HBeAg-positive human serum (mix$^+$ serum). Sera of chronically HBV-infected patients were obtained from venous blood by centrifugation and were pooled. The HBsAg-, HBeAg-positive human serum (the mix$^+$ serum) obtained in this way contained 1.9 x 10^7 infectious HBV particles per ml as determined by Amplicor HBV Monitor (Roche Diagnostic System). Aliquots were kept at -80°C before use. The same protocol was used for the preparation of control serum from healthy donors (the mix$^-$ serum).

PBMCs preparation. PBMCs from a healthy donor (with normal alanine aminotransferase level and negative for HBV DNA in serum and PBMCs based on PCR analysis) were isolated from venous blood collected in the presence of 3.6% trisodium citrate) by centrifugation in Histopaque-1077 (Sigma-Aldrich) gradient. The collected cells were washed twice with a washing medium consisting of RPMI-1640 medium (Gibson BRL) supplemented with 25 mmol/l HEPES, 10% of fetal calf serum, penicillin (100 U/ml) and streptomycin (50 µg/ml). Cell viability was assessed by a trypan blue exclusion test. During all manipulations the condition of cells was monitored by microscopy.

PBMC cultures. RPMI-1640 medium (Gibco BRL) supplemented with 25 mmol/l HEPES, 10% of fetal calf serum, interleukin-2 (20 U/ml), PHA (5 mg/ml), penicillin (100 U/ml) and streptomycin (50 µg/ml) was used for growth of PBMCs. Freshly isolated PBMCs were suspended in the growth medium in a concentration of 1 x 10^6/ml and distributed into 24-well microtiter plates. If necessary, IL-6 was added to a culture in final concentration of 450 ng/ml. The plates were incubated at 37°C in a humidified atmosphere containing 5% CO_2.

In vitro infection. Three-days old PBMC cultures were infected with HBV (using 1.8 ml per well of the mix$^+$ serum diluted 1:1) at 37°C for 90 mins in a 5% CO_2 shaking incubator. For a non-infected control the mix$^-$ serum was used. After incubation the cells were washed three times and the last washing medium was checked for HBV DNA by PCR. Cells were harvested for DNA isolation on days 1, 4, 7 and 10 post infection (p.i.). Culture medium was collected every day p.i. and analyzed.

Cell proliferation. Cell proliferation was measured by a methabenzthiauron (MTT) assay (Hansen *et al*., 1989). The cells for assay were collected from cultures every 24 hrs in triplicate. After 2 hrs of incubation with MTT the cells were lysed and A_{490} was measured in an ELISA scanner. The cell proliferation was calculated as the ratio of A_{490} of tested (infected, with/without the IL-6) to that of A_{490} of control (non-infected) cells.

Statistical analysis. Data representing the means of values from 3 independent experiments are presented as means + error of means. The statistical significance of differences was tested by Student's *t*-test using the Statgraphics Plus software (Statistical Graphics Corp.). A difference with $P \leq 0.05$ was considered significant.

DNA extraction. Total DNA was isolated from cell and medium samples (1 x 10^6 cells/1.5 ml) by phenol-chloroform extraction followed by ethanol precipitation. Medium or washing medium was concentrated 10-fold before DNA isolation.

PCR. The reaction mixture (50 µl) contained 1–10 µg of DNA template in 10 mmol/l Tris-HCl, pH 8.8 supplemented with 1.5 mmol/l $MgCl_2$, 50 mmol/l KCl, 1% Triton X-100, 1 U of Taq polymerase DyNAzyme™ (Finnzymes), 0.2 µmoles of each dNTP and 0.2 µmol/l of each oligonucleotide primer (Chapel *et al*., 1995). The primers specific for the HBV genome: HBP1 (5'-ATCTTCTT ATTGGTTCTTCT-3', nt 430–449) and HBP2 (5'-GTTAG GGTTTAAATGTAATAC-3', nt 845–826). The nucleotide positions are related to the HBV adw subtype with the numbering starts at the unique *Eco*RI site. Primers specific for the beta-globin gene: PG1 (5'-ACACAACTGTGTTCACTAGC-3') and PG2 (5'-CAAC TTCATCCACGTTCACC-3').

The reaction conditions were as follows: 94°C/3 mins, 40 cycles of 94°C/40 secs, 55°C/40 secs, and 72°C/40 secs, and 72°C/10 mins. An Uno II thermal cycler (Biometra) was employed. Each PCR assay was run with at least two negative (uninfected PBMCs) and two positive controls to prevent false-negative and false-positive results. PCR products were analyzed by polyacrylamide gel (10%) electrophoresis. The products of Puc19 digestion with *Msp*I were used as DNA size markers (501, 489, 404, 331, 242, 190, 147, 111, 110, 67, and 34 bp).

Semi-quantitative PCR. Quantity of HBV DNA was determined in total DNA isolated from experimental samples using two different procedures: (i) a limited dilution PCR assay and (ii) a competitive PCR (Sidorkiewicz *et al.,* 2003).

(i) Samples were diluted 2.5, 5, 10, 50, 250 times, respectively and were subjected to PCR with HBV-specific primers. In the same samples the presence of DNA capable of amplification was determined by PCR with the beta-globin-specific primers.

(ii) Briefly, 3 different amounts (0.05, 0.5 and 5 pg) of a competitive HB vector were added to the tested samples (1 x 10^6 cells) prior to the total DNA extraction step. The extracted DNAs originating from cells and containing the HB vector were subjected to PCR with HBV-specific primers HBP1, 2. The HBV DNA concentration (load) was estimated based on PCR product yield: a product of 416 bp originating from viral template and a product of 306 bp originating from the HB vector. The HBV DNA load was calculated using relations: $A_{260} = 1.00$ for 50 µg/ml, M_r of 1 bp = 660, and 1 mol contains 6.022 x 10^{23} molecules.

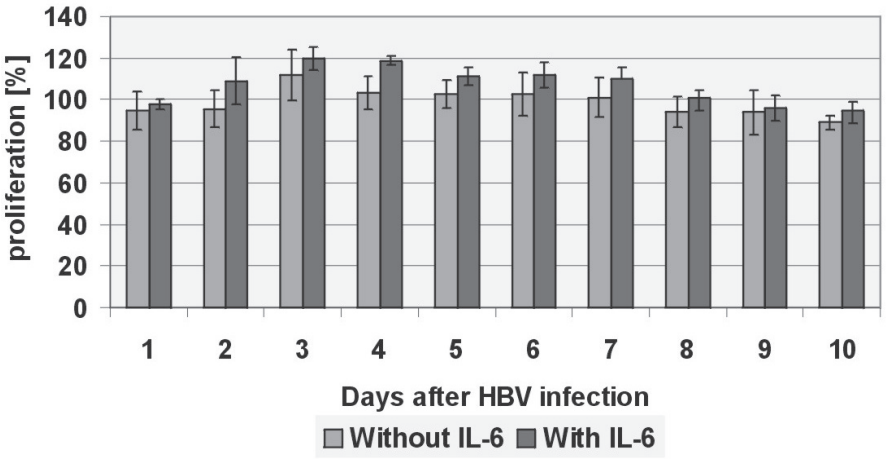

Fig. 1

Eeffect of HBV infection and IL-6 on PBMCs proliferation

The differences for the days 2–7 p.i. were statistically significant.

Results

Cell proliferation: effect of HBV infection and IL-6

The influence of HBV infection and IL-6 on PBMCs proliferation was investigated using the MTT test. Cell proliferation expressed in % of control was estimated for each day during 10 days p.i. The results showed (Fig. 1) that the infection slightly decreased the level of PBMCs proliferation on the first two days p.i. However, on the days 3–7 p.i. an increased level of proliferation was observed. As for the possible effect of IL on the PBMCs proliferation the latter was constantly elevated during whole experiment.

HBV DNA in cells and medium: effect of HBV infection and IL-6

HBV DNA in total DNA isolated from PBMCs and growth medium on the days 1, 4, 7 and 10 p.i was subjected to PCR analysis. A HBV-specific PCR product of 416 bp was detected in the HBV-infected cells throughout the infection regardless of the presence or absence of IL-6 (Fig. 2A). Concerning the amount or the band intensity of the PCR product it increased with the time p.i. We conclude that the presence of IL-6 led to an increased level of HBV DNA in the cells.

Regarding the HBV DNA in medium it was detected on the days 7 and 10 p.i. only (Fig. 2B). The specific band intensity was higher with IL-6 than without it.

Next the qualitative detection was substituted by a semi-quantitative assay of HBV DNA, namely by the limited dilution PCR and competitive PCR (see Materials and Methods). In the limited dilution PCR a beta-globin-specific PCR was included with a corresponding product of 110 bp (Fig. 3, Table 1). The results showed the presence of HBV DNA in PBMCs in the 2.5-fold dilution on the days 1 and 4 p.i. regardeless of IL-6; however, the 5-fold dilution was positive only for the cells exposed to IL-6. On the day 7 p.i. HBV DNA was detected in PBMCs in the 2.5-fold and 5-fold dilutions regardless of IL-6 but in the 10-fold and 50-fold dilutions in the presence of IL-6 only. On the day 10 p.i. the results were similar to the previous ones except that the highest HBV-DNA-positive dilution for the Il-6-exposed cells was only 10-fold (not 50-fold).

Table 1. Detection of HBV DNA

	Dilution of total DNA					
Day p.i.	IL-6	2.5x	5x	10x	50x	250x
1	+	+	+	–	–	–
	–	+	–	–	–	–
4	+	+	+	–	–	–
	–	+	–	–	–	–
7	+	+	+	+	+	–
	–	+	+	–	–	–
10	+	+	+	+	–	–
	–	+	+	–	–	–

In competitive PCR a product of 416 bp originating from viral template and another product of 306 bp originating from the HB vector were present in electrophoretic gels (Fig. 4A). Based on this assay we calculated the HBV DNA content of 1 x 10^6 PBMCs (average from 4 independent experiments). This value varied during infection (cultivation of PBMCs

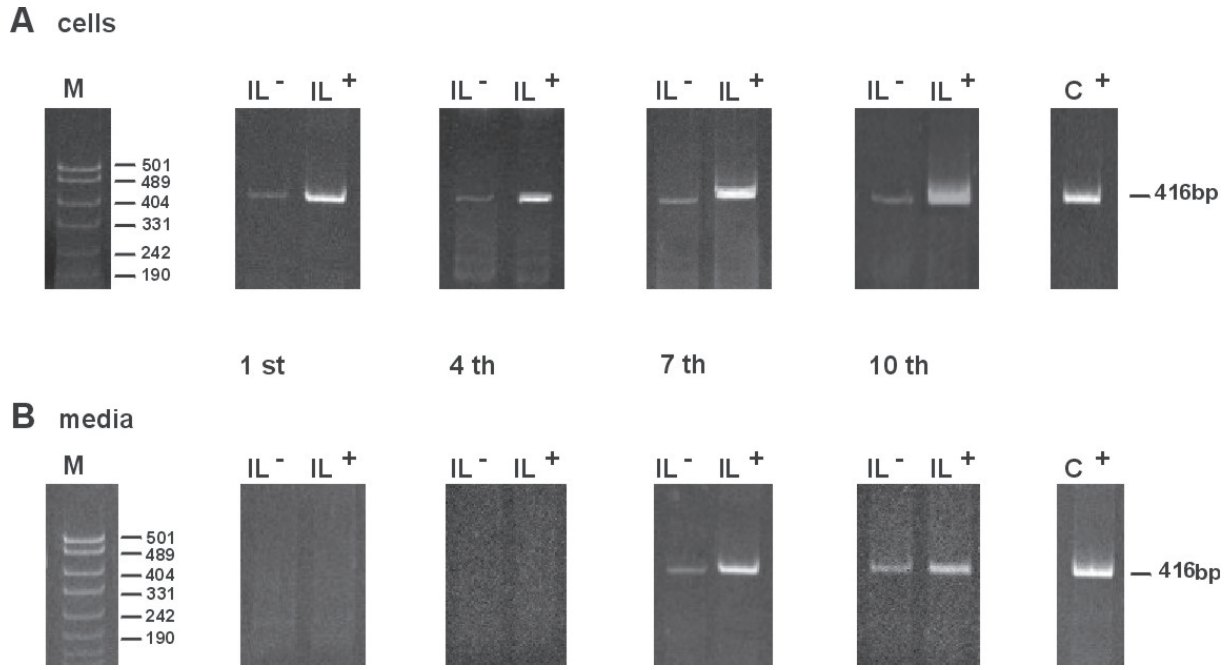

Fig. 2

Efffect of HBV infection and IL-6 on HBV DNA in PBMCs (A) and culture medium (B)

Positive control (C^+). DNA size markers (M).

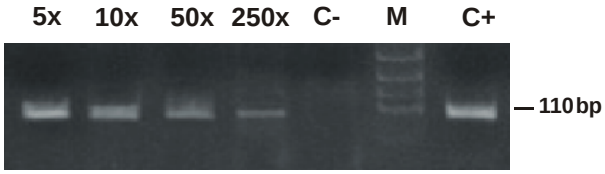

Fig. 3

Semi-quantitative (a limited dilution) detection of ß-globin-specific PCR product of 110 bp

Samples diluted 5x, 10x, 50x and 250x (lanes 5x–250x). Positive and negative controls (lanes C+ and C-). DNA size markers (lane M).

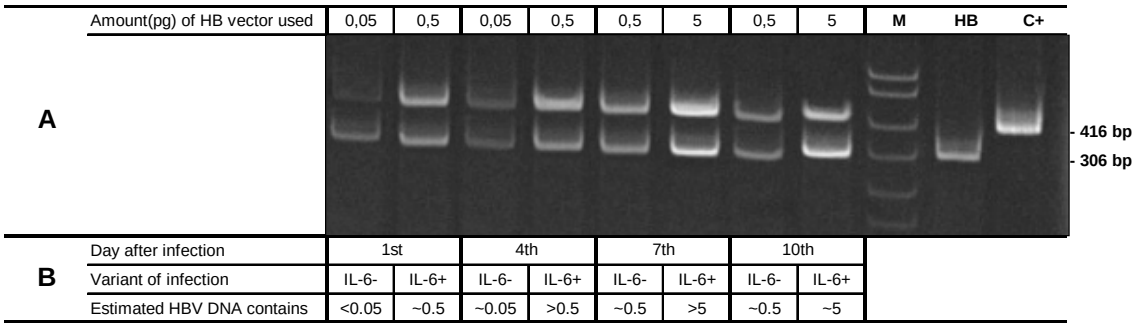

Fig. 4

Semi-quantitative detection (a competitive PCR) of HBV DNA and the effect of IL-6

A. Electrophoresis of PCR products of the HB vector added in different amounts: 0.05, 0.5 and 5 pg. B. Estimated HBV DNA content in PBMCs infected with HBV in the presence or absence of IL-6.

p.i.) from less than 0.05 pg on the day 1 to 0.5 pg on the day 10 p.i. for HBV-infected PBMCs without exogenous Il-6. For the PBMCs infected in the presence of IL-6 the HBV DNA content varied from 0.5 pg on the day 1 to 5 pg on the day 10 p.i. (Fig. 4B). For both cases given above the HBV DNA content was increasing p.i.

Discussion

The early stages of HBV infection as well as cellular receptor(s) involved in HBV entry into target cells are still unknown (Paran *et al.,* 2001). Current data indicate that despite high hepatotropism HBV could be detected in different extrahepatic cells including PBMCs (Roisman *et al.,* 1994). To evaluate the influence of Il-6 on the infection of cells with HBV we chose PBMCs because (i) the adsorption of HBV to PBMCs seems to be a receptor-mediated process (Neurath *et al.,* 1990; Pontisso *et al.,* 1991), (ii) PBMCs were shown to express IL-6 α and β receptors on their surface (Peters *et al.,* 1998), and (iii) these cells are well available. In this study the comparison of HBV-infected and non-infected PBMCs regarding their proliferation confirmed the general observation that there is no cytopathic effect of HBV (Rapicetta *et al.,* 2002). Moreover, starting with the day 3 p.i. we could observe an increased level of proliferation of infected cells. IL-6 caused an elevated cell proliferation between the days 2 and 7 p.i.

This suggested that Il-6, known as an important factor of proliferation in regenerating liver (Galun and Axelrod, 2002) could also induce PBMCs proliferation in both cases: when it was added as an exogenous substance and as a component of HBV-positive sera, known to have a higher IL-6 level than the sera from healthy donors (Tangkijvanich *et al.,* 2000).

We used an *in vitro* infection assay by inoculating cells with a pool of HBV-positive sera and we have determined the level of infection by PCR, the most sensitive method for this type of experiment (Stoll-Becker *et al.,* 1997; Cabrerizo *et al.,* 2000, 2002). To exclude the possibility of false positive results associated with the presence of HBV-positive serum contamination, in each experiment the cells were intensively washed after inoculation with HBV-positive serum and the washing media were always analyzed for the presence of HBV DNA. We were able to detect HBV DNA in PBMCs from the day 1 p.i. Adsorption of HBV particles onto the surface of cells cannot be the only mechanism involved, because in all experiments we observed the increase of HBV DNA content of cells p.i. Overall, the results of the two semi-quantitative assays of HBV DNA were consistent and showed that the amount of HBV DNA in cells was increasing more than 10 times from the day 1 until the day 10 p.i. Addition of IL-6 to the virus inoculum always resulted in the increase in HBV DNA content of PBMCs, which was estimated to be 10 times higher than that of control cells. On the other hand, IL-6 added to the growth medium only had no effect.

Thus, we did not observe any influence of IL-6 on HBV replication, which was the case of hepatoma cell lines (Ohno *et al.,* 1999; Waris and Siddiqui, 2002).

Based on our results we suggest that the presence of IL-6 in the first step of HBV infection is of greatest importance for the infection efficiency. It means that the HBV DNA content of PBMCs correlates at most with the amount of viral particles able to enter the cells during infection and the IL-6 seems to mediate this process.

Our data suggest that HBV may not only be taken up but they also may be replicated by PBMCs and that human IL-6 is able to increase the efficiency of PBMCs infection with HBV *in vitro*. Thus, this type of cell culture and virus can be used as a future model for a more detailed study of the HBV entrance into cells.

Acknowledgements. The authors thank Dr. R. Idem Somiari, Windber Research Institute, Windber, PA, USA for helpful comments, and Mrs. M. Szemraj, SANEPID, Lodz, Poland for her assistance in the collection of sera. This study was supported by the KBN Research Grant 4.PO5A.15.

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