Demonstration of woodchuck hepatitis virus infection of peripheral blood mononuclear cells by flow cytometry and polymerase chain reaction

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Peripheral blood mononuclear cells (PBMCs) from 10 woodchuck hepatitis virus (WHV)-infected woodchucks were examined for the presence of WHV surface (WHs) and core (WHc) antigens (WHsAg and WHcAg) by cytofluorometry using fluorescein isothiocyanate-conjugated anti-WHs and anti-HBc-purified immunoglobulins from woodchuck and human sera. The presence of viral DNA and RNA was detected in the serum and PBMCs from the same blood samples by polymerase chain reaction (PCR) with two primer sets located in the S and C genes of the WHV genome. Seven animals were found positive for both WHsAg and WHcAg on the surface of PBMCs: four WHV-chronic carriers, two WHsAg-positive animals with acute WHV infection, and one woodchuck which was bled during the incubation phase of WHV infection and which became WHsAg-positive only 1 month later. Sixteen to 71% of the studied leukocyte population expressed WHsAg with a low density of expression whereas 7 to 72% expressed WHcAg with a high density of expression. Only two cases were positive for WHsAg without WHcAg on PBMCs, one WHV chronic carrier and one anti-WHs-positive animal. All woodchucks positive for WHcAg and/or WHsAg by cytofluorometry were positive also for WHV DNA and RNA in PBMCs by PCR. The tenth animal was found negative for both viral antigens as well as for WHV DNA and RNA in PBMCs despite the presence of persistent viral DNA in the serum as detected by PCR. Five healthy woodchucks devoid of WHV serological markers served as negative controls. These results obtained with a novel approach further confirm, in the woodchuck model, that a significant proportion of PBMCs are probably permissive for WHV replication. The possible immunopathogenic implications of the phenomenon are discussed.

Introduction

Human hepatitis B virus (HBV), like woodchuck hepatitis virus (WHV), another member of the hepadnavirus family, are mainly hepatotropic and can induce chronic liver disease leading to cirrhosis and hepatocellular carcinoma (Hoofnagle, 1984; Tiollais et al., 1985). The viral genome, however, can also be detected in mononuclear cells from patients with serological evidence of infection and liver disease (Elfassi et al., 1984; Pontisso et al., 1984; Yoffe et al., 1986). In those cases, both free and integrated forms of HBV DNA were observed after Southern blot analysis. Furthermore, WHV DNA sequences have been detected in kidney, pancreas, ovary and testis suggesting that a variety of tissues and organs are susceptible to WHV infection (Korba et al., 1989a).

To demonstrate that the virus can replicate in these haematopoietic cells, viral transcriptional activity was investigated and the presence of HBV RNA species was demonstrated in human peripheral blood mononuclear cells (PBMCs) by in situ hybridization (Hadchouel et al., 1988), Northern blotting (Lobbiani et al., 1990) and polymerase chain reaction (PCR; Baginski et al., 1991). This was also demonstrated for PBMCs of experimentally infected chimpanzees and woodchucks (Korba et al., 1986). Furthermore, Korba et al. (1988, 1989b) demonstrated the ability of lipopolysaccharide to stimulate PBMCs of chronically infected woodchuck to produce complete infectious virions.

A complete assessment of the interplay of hepadnavirus and leukocytes in infected hosts could help in a better understanding of their role in the pathogenesis of infection (Lamelin & Trepo, 1990). Preliminary data indicated that only few mononuclear cells were infected (Shen et al., 1987) and that lymphocyte infection by either HBV or WHV seemed transient. To investigate further the possible infection of PBMCs by WHV in acute and chronic infection, we have used, for the first time in this study, two powerful and sensitive methods: cytofluorometry (Watson, 1987; Wyllie, 1987) to look for the expression of WHs and WHc antigens on PBMCs, and PCR to demonstrate the presence and the translation of the WHV genome in those cells.
Methods

Animals. Fifteen woodchucks, from our colony of *Marmota monax* which originated from Pennsylvania, U.S.A., were made available for this study. They were kept indoors (20 to 25 °C), maintained in separate cages, and fed with rat food supplemented with carrot roots and water ad libitum (Frommel et al., 1984). Among the 15 animals studied, 10 were naturally infected woodchucks [five were chronic WHV-carrying woodchucks (W440, W2047, W2051, W2056 and W2062), five which originated from Pennsylvania, U.S.A., were made available for ad libitum developed self-limited infection (W429, W430, W431, W433 and were positive for WHsAg in the serum, two had seroconverted to anti- W458, W459 and W460). Of the five cases with acute infection two were positive for WHsAg in the serum, two had seroconverted to anti-WHs and one was in the incubation phase but still serologically negative for WHV antigens. It became WHsAg-positive 1 month later (see Results).

Serological assays. Ten ml of peripheral blood was collected from each animal. Six ml of blood was added to 100 units of preservative-free heparin. Serum was obtained from the remaining 4 ml of blood and stored at −20 °C. Sequential samples were tested in parallel in DNA or RNA by PCR as described below, and for WHsAg by radioimmunoassay (RIA) using HbsAg commercial kits, available from Abbott Laboratories, taking advantage of the cross-reactivity between WHsAs and HbsAs (Hantz et al., 1983; Frommel et al., 1984).

Isolation of mononuclear cells from blood. Fresh heparinized venous blood was diluted with Hanks’ balanced salt solution (HBSS), and separated on Ficoll-Isopaque (Pharmacia) by centrifugation at 350 g for 25 min. The mononuclear cells were collected and then washed three times with HBSS (300 g).

Detection of WHsAg in cell lysates. This was carried out as described for HbsAg by Parvez et al. (1987).

Dot blotting. This was performed as described previously (Baginski et al., 1991), using a purified WHV DNA probe labelled by nick translation to a specific activity of 2 × 10⁶ c.p.m./µg (Maniatis et al., 1982).

RNA and DNA samples preparation for PCR reaction. DNA and RNA extraction from serum and PBMCs was performed as previously described (Baginski et al., 1990). RNA was prepared from PBMCs by lysing the cells in cold isotonic buffer, i.e. 10 mM-Tris-HCl and 1:5 mM-MgCl₂, pH 8, 140 mM-NaCl containing 0.5%, NaPO₄ and 0.01% diethylypyrocateonate. The nuclei were pelleted by microcentrifugation. The supernatant was incubated for 10 min at 37 °C and 10 min at 90 °C and microcentrifuged for a few seconds.

After centrifugation, 10 µl of the supernatant was used for reverse transcription (RT)/PCR reactions after DNase I treatment in the presence of RNasin for 30 min at 37 °C.

RT. Total RNA was treated first with RNase-free DNase for 30 min at 37 °C and then with RNase H for 30 min at 37 °C. After inactivation of the enzyme by incubation for 10 min at 95 °C, cDNA synthesis was performed with an oligo(dT) primer for 1 h at 42 °C as previously described (Kawasaki, 1990).

PCR amplification and analysis of amplified DNA. Approximately 1 µg of PBMC DNA or 10 µl of serum DNA was analysed with two sets of oligonucleotide primers: MD03/MD06 in the S region (probe MD09) and MD25/MD27 in the C region (probe MD28) of the hepadnaviruses (Baginski et al., 1990).

The amplification was performed for 30 cycles as previously described using Taq polymerase (Cetus Corporation) and an automated DNA thermal cycler (Perkin-Elmer Cetus) (Saiki et al., 1988). The analysis of the amplified product has been described elsewhere (Lobbiani et al., 1990).

Immunofluorescence techniques. Cells were divided into aliquots (0.5 × 10⁶ to 10⁶ per well) and washed three times in cold PBS (Biomérieux) supplemented with 1% bovine serum albumin (BSA) (Biogenia Lemania) and 0.1% sodium azide (Prolabo). The cells were incubated for 30 min at 4 °C with the appropriate quantity of antibody (at saturating concentration), then washed three times in cold PBS and brought to a concentration of 10⁶ cells/ml in a 1% formaldehyde saline solution. Anti-WHs was obtained from a woodchuck which had recovered from WHV infection and was subsequently boosted with purified WHsAg (Hantz et al., 1983). This serum showed anti-WHs reactivity even at dilutions beyond the anti-WHc endpoint as demonstrated by immunofluorescence analysis of WHV-infected liver (Frommel et al., 1984). Human anti-HBc from a chronic carrier of HBV with high anti-HBc titre antibodies was used in this study. A fluorescein isothiocyanate (FITC)-conjugated woodchuck IgG preparation with a high anti-WHc titre was compared to the human anti-HBc conjugate and was found to be less efficient in detection of WHcAg on the cell surface. Purified immunoglobulins were prepared from anti-WHs and anti-WHc reactive sera and directly conjugated with FITC. The negative control was obtained by incubation with FITC-conjugated immunoglobulins, from the serum of a healthy woodchuck (devoid of all WHV markers).

For neutralization experiments, serum-purified WHsAg (Hantz et al., 1983) and purified recombinant HBcAg (Biogenia Lemania) were used. Undiluted conjugated antibodies (1 mg/ml) were incubated for 30 min at 4 °C with different concentrations (10, 1 and 0.1 µg/ml) of their corresponding antigens or with the same volume of PBS and centrifuged for 10 min at 14000 r.p.m. The supernatant was diluted to the working concentration (0.1 mg/ml) and tested on positive PBMCs.

Fluorescence-activated cell sorter (FACS) analysis. The morpho-immunological analysis of PBMCs was performed using a FAC scan (Becton-Dickinson) equipped with a laser. Among PBMCs, lymphocyte cells were electronically 'gated' following positive identification by SSC-FSC (right angle scatter and forward angle scatter) parameters. Fluorescence intensity was measured on logarithmic scales. Ten-thousand events were recorded by using the List Mode of a CONSORT 30 program. The histograms shown are a representative image of the overall data. Two parameters were used: the percentage of labelled cells on the y axis and the mean fluorescence intensity (correlated with antigenic density on the cell surface) on the x axis. The percentage of positive cells was determined by setting the lower limit above that of the negative control.

Statistical analysis. The correlation between the different detection methods was established using the chi-square test with Yates' continuity correction, and P < 0.05 was considered statistically significant.

The two-tailed paired Student’s t-test was used to evaluate the significance of differences between the two antigenic markers. Results are expressed as the mean ± s.e.m.

Results

PBMCs from WHV-infected and uninfected woodchucks were isolated by Ficoll gradient centrifugation (Table 1). Homogeneous lymphocyte cell subsets were selected by their morphological parameters and further analysed for the presence of WHV-associated antigens. As illustrated in Fig. 1, cytofluorometry allowed the detection of WHV surface and core antigens on the surface of PBMCs from infected woodchucks whereas no
WHV RNA and antigen detection in PBMCs

Fig. 1. Expression of the WHVAg(s) among the lymphocyte cell subset according to their morphological characteristics. (a) PBMCs from an uninfected animal; (b) PBMCs from an acute hepatitis case. Two parameters were used: the percentage of labelled cells on the y axis and the antigenic density (correlated with the fluorescence intensity) on the x axis. (A) Autofluorescence of cells. Cell labelling was performed as described in Methods with (B) the negative control, (C) with anti-WHs and (D) with anti-HBc.

Table 1. Detection of WHV in PBMCs of chronic carrier woodchucks*

<table>
<thead>
<tr>
<th>PCR WHV</th>
<th>RIA</th>
<th>Flow cytometry†</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>RNA</td>
<td>WHsAg</td>
</tr>
<tr>
<td>W440</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>W2047</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>W2051</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>W2056</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>W2062</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* WHV DNA and RNA were detected by PCR using S gene primers; WHsAg was detected by RIA and cytofluorometry; WHcAg was detected only by cytofluorometry.
† Percentage of positive cells: 5<+<20; 20<++<40; ++>40.

signal was detected among uninfected animals. To ensure that immunofluorescence was specific for WHV antigens, different control experiments were performed. To assess the specificity of the anti-WHs and anti-HBc results both antibodies were preincubated with the related antigen.

As shown in Fig. 2, these pretreated antibodies no longer bound to any of the positive samples. Mixing leukocytes from normal animals with serum from a chronic carrier of WHV (W2047) did not yield positive results (data not shown). Finally, WHsAg and WHV DNA could no longer be detected by RIA or PCR in the last wash of PBMCs from the different woodchucks positive for WHsAg and WHV DNA in the serum (data not shown).

PBMCs obtained from five WHV chronic carrier woodchucks were screened by PCR with primers located in the S gene. All were found positive for WHV DNA and RNA (Table 1). The results obtained for W440 (Fig. 3) are representative of this group. The presence of both WHs and WHc antigens was then studied on PBMCs by cytofluorometry. As shown in Table 1, expression of WHsAg on leukocytes was found in all animals, with values of WHsAg-positive cells ranging from 25 to 36%
Fig. 3. Southern blot analysis of PCR products (128 bp) located in the S gene (primer MD03/MD06). The molecular size marker was HaeIII-digested φX174. Lanes: 1, W429; 2, W430; 3, W431; 4, W433; 5, W440.

Fig. 4. Cytofluorometric study of PMBCs from W2062, a chronic carrier of WHV. Two parameters were used: the percentage of labelled cells on the y axis and the antigenic density (correlated with the intensity fluorescence) on the x axis. (A) Autofluorescence of cells. Cell labelling was done as described in Methods with (B) the negative control, (C) anti-WHs and (D) anti-HBc.

Table 2. Detection of WHV DNA and RNA in woodchucks positive for WHsAg in the serum during acute WHV infection*

<table>
<thead>
<tr>
<th>Serum</th>
<th>PBMCs</th>
<th>PCR WHV DNA</th>
<th>WHV DNA</th>
<th>WHV RNA</th>
<th>WHsAg</th>
<th>WHcAg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>WHV DNA</td>
<td>WHV DNA</td>
<td>WHV RNA</td>
<td>WHsAg</td>
<td>WHcAg</td>
</tr>
<tr>
<td>W430</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>W431</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+++</td>
</tr>
</tbody>
</table>

* WHV DNA and RNA were detected by PCR; WHsAg was detected by RIA and cytofluorometry; WHcAg was detected only by cytofluorometry.
† Percentage of positive cells: +, <5 to <20; ++, ≥20 to <40; ++++, ≥40.
**WHV RNA and antigen detection in PBMCs**

### Table 3. Detection of WHV in PBMCs and serum of woodchucks before and after acute infection*

<table>
<thead>
<tr>
<th>Serum</th>
<th>PCR WHV DNA</th>
<th>PCR WHV DNA</th>
<th>PCR WHV RNA</th>
<th>RIA WHsAg</th>
<th>Flow cytometry†</th>
</tr>
</thead>
<tbody>
<tr>
<td>W429</td>
<td>Anti-WHs</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+ + + –</td>
</tr>
<tr>
<td>W433</td>
<td>Anti-WHs</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+ + + –</td>
</tr>
<tr>
<td>W434</td>
<td>None</td>
<td>+</td>
<td>+</td>
<td>ND‡</td>
<td>+ + + –</td>
</tr>
</tbody>
</table>

* WHV DNA and RNA were detected by PCR; WHsAg was detected by RIA and cytofluorometry; WHcAg was detected only by cytofluorometry.

† Percentage of positive cells: +, <5 to <20; + +, ≥20 to <40; + + +, ≥40.

‡ ND, Not determined.

significant correlation (P < 0.05) between the presence of DNA or RNA in PBMCs and WHcAg and/or WHsAg detection by cytofluorometry.

### Discussion

The presence of viral DNA in infected human and woodchuck PBMCs is now commonly accepted. By contrast, the presence of specific transcripts and their corresponding viral protein in these cells had remained poorly documented. However, the presence of viral RNA detected by Northern blot analysis in human (Lobbiani et al., 1990), chimpanzee and woodchuck PBMCs (Korba et al., 1986), and the production of viral particles in human bone marrow culture (Zeldis et al., 1986) as well as mitogen-stimulated woodchuck lymphocyte cultures (Korba et al., 1988, 1989b) strongly suggest that a complete viral life-cycle can occur in these cells. Our results further confirm this lymphotropism and show that woodchuck PBMCs are at least partly able to support viral replication. This is illustrated by the simultaneous detection of viral RNA and both WHsAg and WHcAg on infected PBMCs.

The PCR methodology used in this study was the same as that previously described (Baginski et al., 1991) and excludes the possibility of artefactual detection of RNA by PCR due to DNA or DNA–RNA hybrid contamination as suggested by Hadchouel et al. (1988). As for the detection of viral antigens in PBMCs by cytofluorometry, the following experiments proved their specificity. WHV antigens could not be detected on uninfected woodchuck PBMCs. The possibility of passive absorption of the viral antigens on the cell surface was ruled out by the negative results obtained in experiments involving preincubation of control PBMCs with the serum from a WHV chronic carrier woodchuck. Further-
represent 20% of PBMCs whereas monocyte and
polynuclear cells accounted respectively for 13% and
60% of the PBMCs. In this case, 25% of the lymphocytes
were found positive for WHsAg and 9% for WHcAg.
Monocytes were found positive for WHsAg in only 15% of
cells whereas polynuclear cells reacted for WHsAg and
WHcAg in 8 and 16%, respectively. The lymphocyte
population is therefore infected more often than the total
PBMC population.

Of five WHV chronic carrier woodchucks positive for
WHsAg by cytofluorometry, only four were positive for
WHcAg and, for each of them, the mean percentage of
positive cells was lower for WHcAg than for WHsAg: a
possible explanation could be that WHcAg might be
‘hidden’ and eventually shed as circulating immune
complexes after binding with serum anti-WHc. The
finding of WHc antigen on the surface of naturally
infected lymphocytes may have important pathogenetic
implications. As in the liver (Milich et al., 1987; Mondelli et al., 1982), the presence of viral antigens on
the cell surface makes them susceptible to cytotoxic
immune responses. The involvement of this pheno-
menon in the immunopathogenesis of hepadnavirus
infections warrants further studies.

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139.


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