

**High level of rheumatoid factor is associated with viremia of hepatitis B virus in patients with chronic hepatitis B**

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Running title: RF as a marker for HBV associated LPD

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**Abstract:** Hepatitis viruses are causative agents for chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. While these viruses are also associated with lymphoproliferative disorders (LPD) such as essential mixed cryoglobulinemia and B-cell non-Hodgkin's lymphoma, but the pathogenic mechanism remains unclear. Infection of hepatitis C virus had been reported and confirmed to be associated with LPD. In this study, we investigated the correlation between hepatitis B virus (HBV) infection and LPD. Eighty-four patients with chronic hepatitis B (CH-B) were enrolled. Then markers for LPD, e.g. cryoglobulinemia, high levels of rheumatoid factor (RF), hypocomplementemia, and B cell clonality, were determined and analyzed correlation with viral factors. Results showed that high level of RF was observed in 39.5% of patients with CH-B. High level of RF was not associated with abnormality for the other LPD markers, but with presence of HBV DNA in those patients. The factor of under therapy with the nucleotide analogue was also associated with the high RF. In two patients with CH-B, decreased levels of RF were observed during the antiviral therapy. In conclusion, high level of RF is associated with viremia of HBV in patients with CH-B. Infection with HBV also plays an important role for genesis of LPD in patients with viral hepatitis.

**Key words:** RF: rheumatoid factor, cryoglobulinemia, HBV: hepatitis B virus, LPD: Lymphoproliferative Disorders, B cell clonality

## 1 Introduction

2  
3 Infection with hepatitis viruses causes a variety of extra hepatic manifestations, e.g.  
4 cryoglobulinemia, malignant lymphoma, glomerulonephritis. Especially  
5 lymphoproliferative disorders (LPD) and autoimmune diseases are common  
6 abnormalities among such patients infected with hepatitis virus <sup>1)</sup>. Prevalence of HCV  
7 and HBV carrier was 10.1% and 7.3% in patients with the non-Hodgkin's lymphoma,  
8 respectively <sup>2)</sup>. These rates were around five-fold higher than that of general Japanese  
9 population. Further more, these abnormalities are observed not only the patients with  
10 active hepatitis but also asymptomatic patients, suggesting that regular examination is  
11 necessary for identify immunologic disorders especially in patients with asymptomatic  
12 viral hepatitis.

13 We previously reported that high prevalence of abnormalities for markers of LPD  
14 are found in patients with chronic hepatitis C <sup>3)</sup>. These abnormalities were associated  
15 with HCV infection and/or adsorption with B cells of the patients. The  
16 cryoglobulinemia, high levels of rheumatoid factor (RF), low complement and clonal  
17 expansion of B cells were frequently observed in patients with chronic hepatitis C.  
18 About 74% of the patients were HCV RNA positive in B cells isolated from those  
19 patients studied. These results suggest that HCV infection plays important roles not only  
20 for liver diseases but also for immunological disorders in the patients. In fact, recent  
21 study also demonstrated that abnormal activation of B cells was observed both in  
22 patients with chronic hepatitis B and C <sup>4)</sup>.

23 In this study, we investigated whether HBV infection also induced LPD and/or  
24 immunologic disorders even in patients with CH-B. Abnormality of markers for LPD  
25 was evaluated in CH-B patients.



## Patients and Methods

### *Patients*

During 2002 through 2013, 84 patients with chronic hepatitis B (CH-B), who were managed in Showa University Hospital, were enrolled. Eleven patients underwent the antiviral therapy using nucleic acid analogues among them. Diagnosis of HBV infection was based on the detection of HBs antigen and HBV DNA in the serum prior to the initiation of therapy. Viral information [genotype of HBV and titer of HBV DNA in serum], host factors [age, gender, platelet counts, serum levels of alanine transaminase (ALT) and  $\gamma$ -glutamyl transpeptidase ( $\gamma$ -GTP)], immunological markers [IgG, IgA and IgM] and markers for LPD [cryoglobulinemia, high levels of rheumatoid factor (RF), hypocomplementemia, and B cell clonality] were analyzed. Clinical characteristics of the CH-B patients are shown in Table 1. The study protocol was approved by the Ethics Committee of Showa University School of Medicine, Tokyo, Japan. Informed written consent was obtained from each participant and the study followed the ethical guidelines of the 1975 Declaration of Helsinki.

### *Markers of Lymphoproliferative Disorders*

Cryoglobulinemia was detected by a semi-quantitative centrifugation method. Briefly, blood samples were centrifuged at 600 x g for 20 min at 37°C. Sera were cooled to 4°C and left to stand for 48 h, and centrifuged again at 2,500 x g for 10 min at 4°C. The emergence of cryocrit at 4°C and its disappearance by warming up to 37°C for 20 min was regarded positive for cryoglobulin. RF was determined by the latex turbidimetric assay. The C3, C4 activities were determined by immune nephelometry, while the CH50 was assayed by the immunoturbidimetric method.

### *Amplification of the $V_H$ Region in Immunoglobulin by PCR and the B cell clonality*

## 1 assay

2 In 63 patients with CH-B, clonal expansion of B cells (B cell clonality) was  
3 determined from the sequence uniformity of complementarity-determining-region 3  
4 (CDR3) of the immunoglobulin heavy chain (Ig-V<sub>H</sub>)<sup>5)</sup> (Fig. 1A). RNA (1  $\mu$ l) from  
5 PBMC which derived from patients with CH-B was reverse transcribed into cDNA and  
6 amplified using the GeneAmp<sup>®</sup> EZ rTth RNA PCR kit (Applied Biosystems, California,  
7 USA), according to the manufacturer's instructions. Amplification was carried out with  
8 FW3 primer (5'- CTG AGG ACA CGG CCG TGT ATT ACT G -3') in the V<sub>H</sub> region  
9 and hM3 primer (5'-GGA AAA GGG TTG GGG CGG AT-3') located 8 nt downstream  
10 from the start of the C<sub>H</sub>1 exon in C $\alpha$  exon in The PCR products were visualized by  
11 staining with ethidium bromide after they had been electrophoresed on 4% agarose gels.  
12 The single band on the agarose gel indicates the presence for clonal expansion of B cells  
13 in the patient (Fig. 1B), while the broad band indicates absence of the clonality.

14

## 15 Statistical Analysis

16 The median of continuous variables, without the normal distribution, was  
17 compared by the Mann-Whitney *U* test. Comparison of discontinuous variables was  
18 performed by the chi-squared test or Fisher's exact test with the JMP ver. 10 software  
19 (SAS Institute, Cary, NC). A *P* value < 0.05 was considered statistically significant.  
20 Values with the normal distribution were expressed as the mean  $\pm$  SE. Data of variables,  
21 not distributed normally, were transformed into log values as required.

22

23

## Results

### *High level of RF was observed in patients with CH-B*

Table 1 showed that high levels of RF were observed. The frequency of patients with high RF ( $\geq 10$  IU/ml) was 39.5%. Low C3 was also observed in 10.3% of patients with CH-B. The B cell clonality was identified only in a patient (1.6%) with CH-B. The data showed that 50.0% of the patients with CH-B had at least one abnormality of LPD markers.

### *Abnormality of RF was not associated with any other markers of LPD but with presence of HBV DNA in sera*

We tried to determine the association factors for abnormality of RF in patients with CH-B. Table 2 showed that no parameter was identified among biochemical, immunologic and LPD markers. As far as viral markers, the HBV DNA titers in patients whose RF was more than 10 IU/ml were statistically higher than those in patients whose RF was less than 10 IU/ml (Table 3). We also investigated the effect of antiviral therapy using the nucleotide analogue against the abnormality of RF. Table 3 also showed that the frequency of high levels of RF ( $\geq 10$  IU/ml) in CH-B patients under the antiviral therapy was lower (6.3%) than that in CH-B patients without antiviral therapy.

### *Correlation between HBV DNA titers and levels of RF in sera of patients*

We next investigated correlation between the HBV DNA titers and levels of RF. Table 4 showed that frequency for high levels of RF ( $\geq 10$  IU/ml) in CH-B patients, whose HBV DNA titers were more than 5.0 log copies/ml, was the same as that of normal levels of RF ( $<10$  IU/ml). On the other hand, frequency for high levels of RF ( $\geq 10$  IU/ml) in CH-B patients, whose HBV DNA titers were less than 3.0 log copies/ml, was statistically lower than that of normal levels of RF ( $<10$  IU/ml).



*Time course of HBV DNA titers and levels of RF in sera of patients during the therapy with nucleotide analogue*

We further investigated effects of antiviral therapy against levels of RF in two patients with CH-B (Figure 2). The two patients (Cases 1 and 2) were daily treated with 0.5 mg of entecavir (ETV) and monitored HBV DNA titers and levels of RF through the therapy. Case 1 is a 20 year-old male whose HBe antigen is positive, while case 2 is a 70 year-old male whose HBe antigen is negative. Figure 2 showed that the level of RF was 33.3 IU/ml and the HBV DNA titer was 5.9 log copies/ml in case 1 before therapy (Case 1). When the HBV DNA reached undetectable at 7 months after the beginning of therapy, the level of RF was less than 7 IU/ml. In case 2, the level of RF was 45.4 IU/ml and the HBV DNA titer was 4.6 log copies/ml before therapy. When the HBV DNA reached undetectable at 40 months after the beginning of therapy, the level of RF was 7 IU/ml. These results confirm that high level of rheumatoid factor is associated with viremia of HBV in patients with chronic hepatitis B.

## Discussion

It has been epidemiologically demonstrated that hepatitis viruse, especially HCV, causes a number of extrahepatic manifestations<sup>6,7)</sup>. Among these manifestations, LPD is most closely related to HCV infection<sup>8)</sup>. Hence, it has been accepted widely that the chronic infection with HCV leads to a clonal expansion of B cells and that the sustained proliferation of B cells that would promote the occurrence of genetic mutations<sup>8)</sup>. HBV also causes a variety of extrahepatic manifestations. The joints, muscle, and skin are the main locations of clinical manifestations. Autoimmune diseases and LPD are also common disorders, e.g. malignant lymphoma and mixed cryoglobulinemia<sup>9)</sup>. Furthermore, asymptomatic disorders for immunologic and LPD markers were observed in patients with CH-B as well as in patients with CH-C. It was reported that 15% of patients had at least one immunologic abnormality, mainly anti-smooth muscle antibodies and anti-nuclear antibodies<sup>10)</sup>. Most of these abnormalities are associated with the disorders of B cells,

The present study has revealed that high levels of RF and low C3 were major abnormalities among patients with CH-B. Especially high levels of RF were observed in 39.5% of patients with CH-B. RF is the antibody against the Fc portion of immunoglobulin G (IgG). High levels of RF are frequently observed not only in patients with rheumatic diseases, but also with non-rheumatic diseases, including mixed cryoglobulinemia, Sjögren's syndrome, mixed connective tissue disease, and even in healthy subjects<sup>11)</sup>. We also reported that 48% and 41% of patients with CH-C and CH-B had abnormality for RF in our small scale of study, respectively<sup>3)</sup>. Another group reported that abnormality of RF was observed in 4% of healthy subjects<sup>12)</sup>. The incidence of abnormal RF was also reported higher in older subjects without rheumatic disease, ranging from 3 to 25 %<sup>13,14)</sup>. Our data confirm that age factor does not affect high levels of RF in our cohort. In the present study, we used the latex turbidimetric



1 assay for determination of RF levels. This method is one of the standard methods, which  
2 were licensed in Japan, to determine the levels of RF. Reference value of the RF was  
3 less than 7 IU/ml in our assay. But this reference value has changed into less than 15  
4 IU/ml in Japan recently. In our previous study to investigate abnormalities for LPD  
5 markers of patients with CH-C, we regarded patients whose RF was more than 10 IU/ml  
6 as high level <sup>3)</sup>. We also regarded patients with CH-B whose RF was more than 10  
7 IU/ml as those with high levels of RF. Frequency of patients, whose RF was more than  
8 15 IU/ml, was 34.6%. This frequency is almost the same as that of patients, which RF  
9 was more than 10 IU/ml.

10 Chronic inflammation of liver by viruses may play an important role for  
11 production of abnormal immunoglobulin, leading to autoimmune diseases and LPD.  
12 Our former report demonstrated that HCV was detected in B cells of patients with CH-C,  
13 and that presence of HCV in B cells was associated with LPD <sup>3)</sup>. There is no report  
14 about HBV replication in B cells. Detail mechanism of immune disorders in patients  
15 with CH-B is still unknown.

16 Next we analyzed the association factors with abnormality of RF, but we could not  
17 identify any host factors by the univariable analysis. Only presence of HBV DNA in  
18 sera, and post-treatment with nucleotide analogue are associated factors with the high  
19 levels of RF. After the antiviral therapy, the HBV DNA titers and levels of RF were  
20 decreased together in patients with CH-B. In patients whose HBV DNA < 3.0 log  
21 copies/ml, only 15.6% of patients had high levels of RF. Figure 2 also directly showed  
22 that the levels of RF reached less than 7 IU/ml when HBV DNA titers became  
23 undetectable in two patients with CH-B. Altogether, obtained results strongly suggest  
24 that viremia of HBV causes high levels of RF in patients. Another viral factor,  
25 difference of HBV genotypes, is not correlated with high levels of RF. This result is

1 consistent with the previous report that HBV genotypes are not associated with  
2 extrahepatic manifestations <sup>10)</sup>.

3 Biologic significance of high levels of RF in viral hepatitis is hard to answer. Our  
4 data do not show the correlation between abnormal RF and presence of the B cell  
5 clonality. The present study apparently indicates that pathogenesis of HBV for  
6 immunologic disorders and LPD is weaker than that of HCV. These differences of  
7 pathogenesis between the two viruses may be occurred by the ethnicity. Further studies  
8 are necessary to clarify molecular mechanisms for generation of extrahepatic  
9 manifestations, LPD and abnormal levels of RF and the correlation with malignant  
10 lymphoma in patients with viral hepatitis.

#### 11 **Conflict of interest**

12 The authors have declared no conflict of interest.  
13  
14

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1    **Legends to the Figures**

2

3    Fig. 1. (A) Scheme of primers used for the B cell clonality assay. The FW3 and hM3  
4    primers were used for amplification of the CDR3 gene. (B) The broad bands, which  
5    molecular sizes were around 300 to 330 base pairs, show negative (lanes 1 to 3), the  
6    single band indicated by the arrow shows positive (lane) 4 for the mono-clonality in the  
7    CDR3 gene, which molecular size should vary in each B cell of healthy adults.

8

9    Fig. 2. Time course for HBV DNA titers and levels of RF in two patients with CH-B,  
10    Cases 1 (A) and 2 (B), after beginning of the antiviral therapy using the entecavir  
11    (ETV).

12

13

14

Table 1 Clinical characteristics of patients with chronic hepatitis B

	HBV CH (n = 84)
Age (years)	47.8±13.0
Gender (M/F)	47/37
Plateletes (10 <sup>4</sup> /μl)	20.5±5.0
ALT (IU/l)	49.8±79.0
γGTP (IU/l)	28.2±2.3
IgG (mg/dl)	1276.8±266.4
IgA (mg/dl)	227.0±107.7
IgM (mg/dl)	118.0±84.4
Cryoglobulinemia	1/80 (3.7%)
RF (>10 IU/ml)	49/81 (39.5%)
C3 (<86mg/dl)	8/78 (10.3%)
C4(<10mg/dl)	1/78 (1.3%)
CH50 (<20U/ml)	0 /78 (0%)
B cell clonality	1/63 (1.6%)
HBV DNA (log copies/ml)	2.39±0.32
HBs antigen > 200 (IU/ml)	61/81 (75.3%)
HBe antibody positive	63/69 (91.3%)
HBV genotype (A/B/C/D/ND)	6/17/32/1/38

**NOTE** Data are no (%) or the mean ± SE.



Table 2 Comparison of the other LPD markers between patients with and without abnormality of RF

	RF<10 IU/ml (n = 49)	RF $\geq$ 10 IU/ml (n = 32)	P value
Age (years)	49.5 $\pm$ 1.9	45.9 $\pm$ 2.3	ns
Gender (M/F)	26/23	17/15	ns
ALT (IU/l)	48.9 $\pm$ 11.1	48.7 $\pm$ 13.7	ns
Plateletes (10 <sup>4</sup> / $\mu$ l)	20.2 $\pm$ 0.7	20.7 $\pm$ 0.9	ns
$\gamma$ GTP (IU/l)	30.1 $\pm$ 3.0	26.4 $\pm$ 3.9	ns
IgG	1238.6 $\pm$ 49.2	1344.7 $\pm$ 63.6	ns
IgA	227.1 $\pm$ 19.9	236.7 $\pm$ 25.7	ns
IgM	117.1 $\pm$ 15.7	120.1 $\pm$ 20.3	ns
Cryoglobulinemia	3/49 (6.1%)	0/31 (0%)	ns
C3 (<86mg/dl)	6/48 (12.5%)	1/28 (3.6%)	ns
C4 (<10mg/dl)	0/48 (0%)	1/28 (3.6%)	ns
CH50 (<20U/ml)	0/48 (0%)	0/28 (0%)	ns

**NOTE** Data are no (%) or the mean  $\pm$  SE. Abbreviations: ns, not significant;

Table 3 Comparison of HBV markers between patients with and without abnormality of RF

	RF<10 IU/ml (n = 49)	RF $\geq$ 10 IU/ml (n = 32)	P value
HBV DNA (log copies/ml)	3.31 $\pm$ 0.29	4.66 $\pm$ 0.35	0.0045*
HBs antigen > 200 (IU/ml)	33/47 (70.2%)	25/31 (80.7%)	ns
HBe antibody positive	39/42 (92.9%)	23/25 (92.0%)	ns
HBV genotype (A/B/C/D)	3/11/21/0	3/6/9/1	ns
Under treatment with nucleotide analogue	18/49 (36.7%)	2/32 (6.3%)	0.0016*

**NOTE** Data are no (%) or the mean  $\pm$  SE. \*Significantly different between the two groups ( $P < .01$ ) by the Fisher's exact analysis. Abbreviations: ns, not significant;

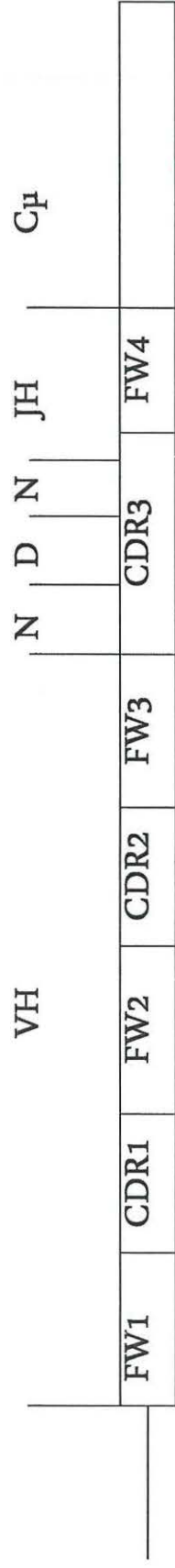
Table 4 Correlation between high titers of HBV DNA and abnormality of RF

	RF<10 IU/ml (n = 49)	RF $\geq$ 10 IU/ml (n = 32)	P value
HBV DNA < 3.0 (log copies/ml)	28/49 (57.1%)	5/32 (15.6%)	0.0002*
HBV DNA $\geq$ 5.0 (log copies/ml)	11/49 (22.5%)	9/32 (28.1%)	ns

**NOTE** Data are no (%). \*Significantly different between the two groups ( $P < .01$ ) by the Fisher exact analysis. Abbreviations: ns, not significant;



(A)



FW3 →  → hM3

(B)

1 2 3 4

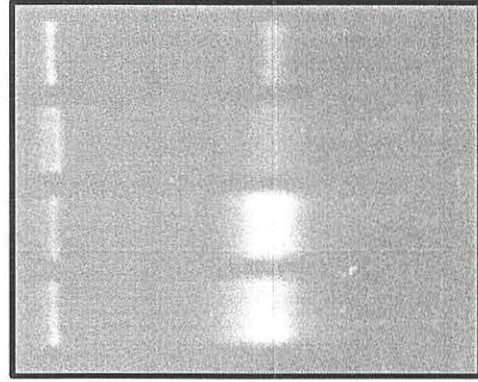


Figure 1

(A) Case 1  
20y, Male  
HBe antigen positive

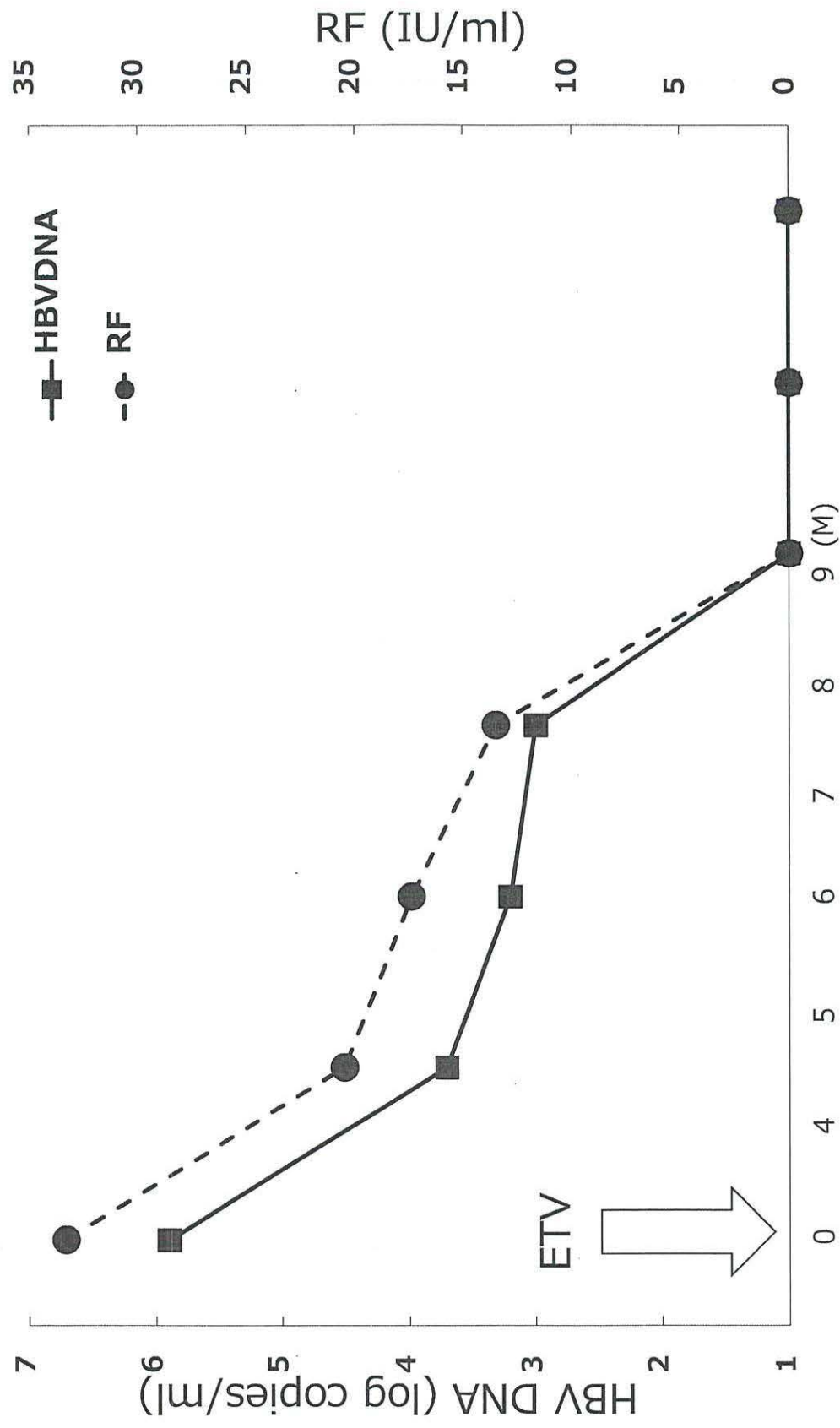


Figure 2

(B) Case 2  
70y, Male  
HBe antigen negative

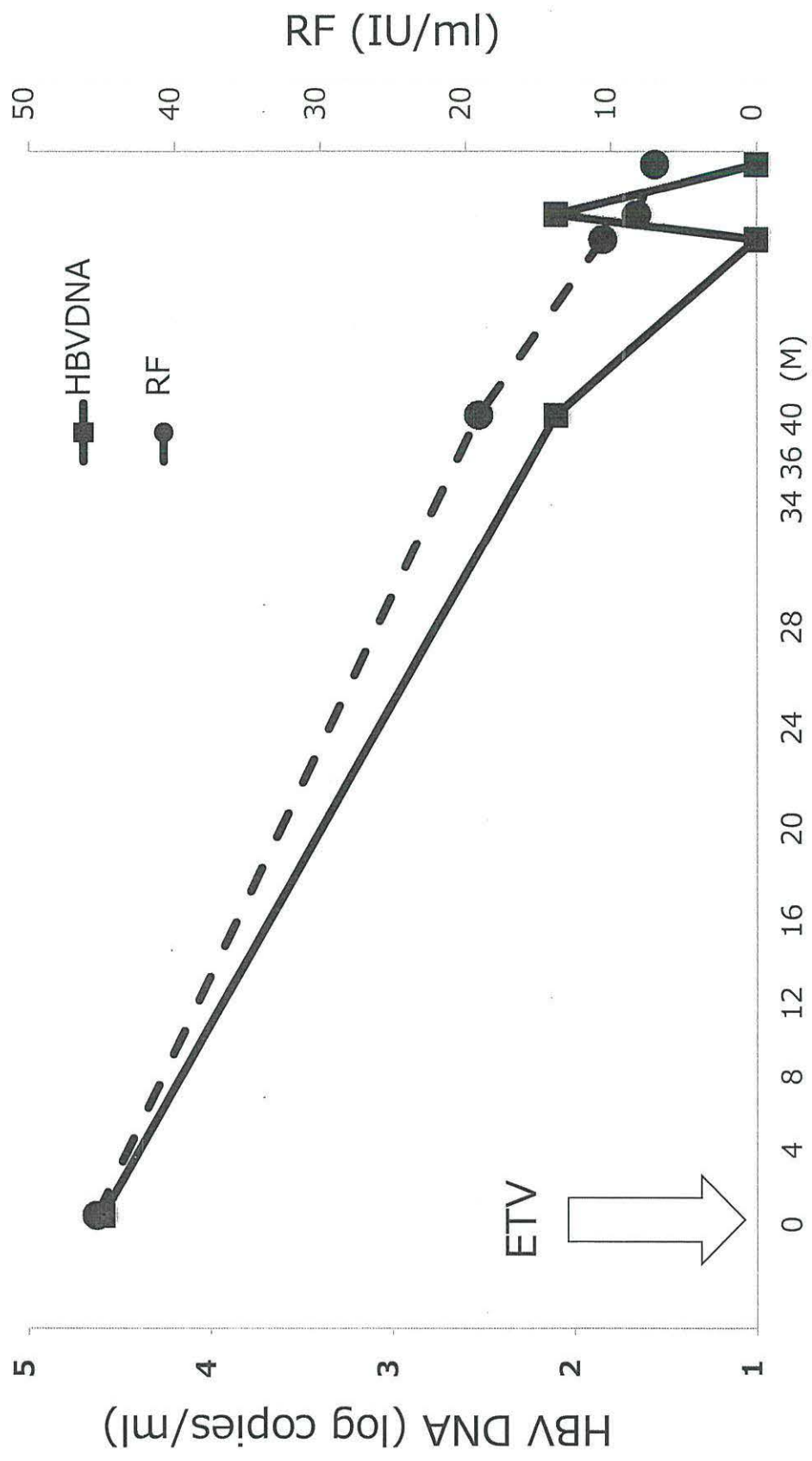


Figure 2