Absence of viral interference and different susceptibility to interferon between Hepatitis B and C virus in human hepatocyte chimeric mice

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Abbreviations:

GAPDH, glyceraldehydes-3-phosphate dehydrogenase; HBeAg, hepatitis B e antigen;

HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCV, hepatitis C virus;

HSA, human serum albumin; IFN, interferon; OAS, 2'-5' oligoadenylate synthetase;

PCR, polymerase chain reaction; SCID, severe combined immunodeficiency; uPA,

urokinase-type plasminogen activator

Abstract

Aim: Both hepatitis B virus (HBV) and hepatitis C virus (HCV) replicate in the liver and show resistance against innate immunity and interferon (IFN) treatment. Whether there is interference between these two viruses is still controversial. We investigated the interference between these two viruses and the mode of resistance against IFN.

Methods: We performed infection experiments of either or both of the two hepatitis viruses in human hepatocyte chimeric mice. Huh7 cell lines with stable production of HBV were also established and transfected with HCV JFH1 clone. Mice and cell lines were treated with IFN. The viral levels in mice sera and culture supernatants and mRNA levels of IFN-stimulated genes were measured.

Results: No apparent interference between the two viruses was seen *in vivo*. Only a small (0.3 log) reduction in serum HBV and a rapid reduction in HCV were observed after IFN treatment irrespective of infection with the other virus. In *in vitro* studies, no interference between the two viruses was observed. The effect of IFN on each virus was not affected by the presence of the other virus. IFN-induced reductions of viruses in culture supernatants were similar to those in *in vivo* study.

Conclusion: No interference between the two hepatitis viruses exists in the liver in the absence of hepatitis. The mechanisms of IFN resistance of the two viruses target different areas of the IFN system.

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1. Introduction

Both hepatitis B virus (HBV) and hepatitis C virus (HCV) infections are serious health problems worldwide. More than 350 and 170 million people are infected with HBV and HCV, respectively [1,2]. Both types of hepatitis viruses result in the development of chronic liver infection and lead to death due to liver failure and hepatocellular carcinoma [3]. To date, interferon (IFN) remains one of the most important drugs available for the treatment of both types of hepatitis viral infections. Although it is assumed that IFN suppresses viral replication through the effect of IFN-induced gene products such as MxA, PKR, and 2'-5' oligoadenylate synthetase [4], the precise mechanism of action of these proteins on both hepatitis viruses are unknown.

Coinfection with both viruses leads to a rapid and sever progression of chronic liver disease [5], with a higher risk of hepatocellular carcinoma [6]. Currently, there is a debate on whether there is interference between the two hepatitis viruses or not, with some favoring such interference [7] and others arguing against such concept [8]. A number of mechanisms can cause interference between viruses. A major mechanism of interference is induction of IFN by one virus to prevent the replication of the second virus. However, viruses develop their own strategy to resist the effect of IFN. In clinical practice, practitioners often feel that reduction of HBV in serum by IFN therapy is poorer compared with HCV. HCV levels in sera of IFN-treated patients decrease relatively rapidly, and a proportion of patients eventually show complete eradication of the virus. Furthermore, the recent use of peg-IFN in combination with

ribavirin has improved the eradication rate [9]. However, eradication of HBV by IFN

is usually difficult even using IFN combined with ribavirin [10].

The mechanisms developed by viruses to resist host innate immunity including IFN

signaling are well established in some viruses. Such mechanisms involve interruption

of IFN signaling by interacting molecules that transduce the signal from IFN receptor

through the Jak-STAT (signal transducer and activator of transcription) pathway [4].

For example, viral proteins of paramixoviruses inhibit IFN signaling [11]. Several

studies have also examined the mechanisms by which HCV resists the host immune

system. These include degradation of Cardif adaptor protein by NS3A/4 protease [12].

In general, expression of HCV protein is associated with inhibition of STAT1 function

independent of STAT tyrosine phosphorylation [13]. In addition, expression of the

HCV core protein in cultured cells is associated with increased expression levels of

the suppressor of cytokine signaling (SOCS)–3 [14]. The NS5A and E2 proteins are

both inhibitors of PKR protein [15]. These strong actions of HCV against innate

immunity are consistent with the high chronicity rate of the virus. However, IFN

effectively reduces HCV replicon in Huh7 cells [16], suggesting that the virus has

only little potential to disturb the actions of IFN.

In contrast to HCV, the mechanisms of IFN resistance by HBV are poorly

understood. To date, only a few studies have reported the molecular mechanisms of

HBV resistance against the actions of IFN. For example, the HBV-related resistance

to IFN involves up-regulation of protein phosphatase 2A (PP2A) as the primary event,

which subsequently leads to inhibition of protein arginine methyltransferase 1

(PRMT1) and reduced STAT1 methylation [17]. In addition to these molecular

mechanisms, microarray analyses of serial liver biopsies of experimentally-infected

110   chimpanzees showed striking differences in the early immune responses to HBV and

HCV. For example, HCV induced early changes in the expression of many

intrahepatic genes, including genes involved in type I IFN response [18] whereas

HBV did not induce any detectable changes in the expression of intrahepatic genes in

the first weeks of infection [19].

115   HBV-HCV double infection is a good model to use for assessment of the

mechanism of IFN resistance by these two viruses because one can test the effect of

IFN on one virus in the presence of the other one. Recently, Bellecave et al. [20]

established a novel *in vitro* model system in Huh7 cells that allowed the analysis of

both viruses in a replicating context, and reported the absence of direct viral

120   interference. To this end, we used human hepatocyte chimeric mice and cell culture

systems in the present study. The results showed that the presence of HBV does not

affect the actions of IFN on HCV and vice versa. These results indicate the lack of

interference between the two viruses in liver cells and that the reported interference

between the two viruses might be via inflammation including death of infected cells

125   by cytotoxic T-cells, cytokines including IFN-α and IFN-γ, and interleukins produced

by hepatocytes and infiltrating T cells.

2. Material and Methods

130   *2.1 Transfection of Huh7 cells with HBV DNA and HCV RNA.*

Huh7 cells were grown in Dulbecco's modified Eagle's medium supplemented with

10 % (v/v) fetal bovine serum at 37°C and under 5% CO_2. Cloning of HBV DNA and

the plasmid construction were performed as described previously [21]. For production

of stably transfected cell lines, Huh7 cells were seeded onto 90-mm-diameter culture

135   dishes. Twenty micrograms of the plasmid pTRE-HB-wt [21] was transfected by the

calcium phosphate precipitation method. Twenty-four hours after transfection, the

cells were split and cultured in Hygromycin B-DMEM selection medium (300 μg/ml;

Invitrogen Japan K.K., Osaka, Japan), while 50 colonies were isolated and cultured

for identification of virus-producing cell lines. Clones positive for both hepatitis B

140   surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) were selected and further

analyzed for production of HBV particles. Finally, three cell lines that produced more

than 10^5 copy/ml of HBV DNA in supernatant were selected and used for further

experiments.

For transfection with HCV RNA, we used pJFH1, which contains the cDNA of

145   full-length genotype 2a HCV clone JFH1 downstream of the T7 promoter [22]. *In*

vitro synthesis of HCV RNA and electroporation into Huh7 cells were performed as

described previously [22, 23]. Briefly, cells were treated with trypsin, washed twice

with ice-cold RNase-free phosphate-buffered saline, and resuspended in Opti-MEM I

(Invitrogen, Carlsbad, CA) at a final concentration of 7.5×10^6 cell/mL. Then, 10 μg

150   of HCV RNA to be electroporated was mixed with 0.4 mL of cell suspension and

subjected to an electric pulse (950 μF and 260 V) using the Gene Pulser II

Electroporation System (Bio-Rad, Hercules, CA). After electroporation, the cell

suspension was left for 5 min at room temperature and then incubated under normal

culture conditions in a 10-cm diameter cell culture dish.

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2.2 Generation of human hepatocyte chimeric mice.

Generation of the uPA$^{+/+}$/SCID$^{+/+}$ mice and transplantation of human hepatocytes

were performed as described recently by our group [21, 23, 24]. All mice were

transplanted with frozen human hepatocytes obtained from the same donor. Infection,

160   extraction of serum samples, and euthanasia were performed under ether anesthesia.

The concentration of serum human serum albumin (HSA), which correlates with the

repopulation index [24], was measured in mice as described previously [21]. All

animal protocols described in this study were performed in accordance with the

guidelines of the local committee for animal experiments. The experimental protocol

165   was approved by the Ethics Review Committee for Animal Experimentation of

Graduate School of Biomedical Sciences, Hiroshima University.

2.3 Human serum samples.

Human serum samples containing high titers of either HBV DNA (5.3 x 10^6

170   copies/mL) or genotype 1b HCV (2.2 x 10^6 copies/mL) were obtained from patients

with chronic hepatitis with a written informed consent. The individual serum samples

were divided into small aliquots and separately stored in liquid nitrogen until use.

Chimeric mice were injected intravenously with 50 µL of either HBV- or

HCV-positive human serum. Some mice were injected with HBV-positive human

175   serum at 6 weeks after injection of HCV-positive human serum.

2.4 Analysis of HBV and HCV.

HBsAg and HBeAg in culture supernatants were measured by commercially available ELISA (enzyme-linked immunosorbent assay) kits (Abbott Japan, Osaka).
180 DNA was extracted from these samples by SMITEST (Genome Science Laboratories, Tokyo, Japan) and dissolved in 20 µL H_2O [21, 25]. RNA was extracted from serum samples by Sepa Gene RV-R (Sankojunyaku, Tokyo), dissolved in 8.8 µL RNase-free H_2O, and reverse transcribed using random primer (Takara Bio Inc., Shiga, Japan) and M-MLV reverse transcriptase (ReverTra Ace, TOYOBO Co., Osaka) in a 20 µL
185 reaction mixture according to the instructions provided by the manufacturer [23]. HCV core antigen in the culture medium was detected with HCV Ag assay (Ortho-Clinical Diagnostics, Rochester, NY).

2.5 RNA extraction and measurement of mRNAs of IFN-induced genes by
190 quantitative RT-PCR.

Total RNA was extracted from cell lines using the RNeasy Mini Kit (Qiagen, Valencia, CA). One µg of each RNA was reverse transcribed with ReverseTra Ace (TOYOBO Co.) and Random Primer (Takara Bio, Kyoto, Japan). We quantified the transcripts for MxA, 2'-5' oligoadenylate synthetase (OAS) and PKR. Amplification
195 and detection were performed using ABI PRISM 7300 (Applied Biosystems, Foster City, CA). Results were normalized to the transcript levels of glyceraldehydes-3-phosphate dehydrogenase (GAPDH).

2.6 Statistical analysis.

Changes in HBV DNA and HCV RNA in mice serum were compared by Mann-Whitney U and unpaired t tests. Differences in HBV DNA and HCV core antigen in mice serum and culture supernatants were analyzed by one-way analysis of variance followed by Scheffe's test. A *P* value of less than 0.05 was considered statistically significant.

3. Results

3.1 Infection of chimeric mouse with HBV and HCV, and their susceptibility to IFN.

To investigate the interference between HBV and HCV and examine the effect of IFN on both of these two viruses *in vivo*, we used six human chimeric mice. Each of six mice was inoculated intravenously with 50 μL of serum samples obtained from either HBV- or HCV-positive patients. The median HBV DNA level in HBV-positive serum-inoculated mice was 1.4×10^8 copies/mL (range 5.3×10^6 - 3.6×10^9 copies/mL) at 6 weeks after inoculation (Fig. 1A) similar to our recent observation [21]. Similarly, the median HCV RNA level in HCV-positive human serum-inoculated mice was 1.0×10^7 copies/mL (range 1.2×10^6 - 0.8×10^7 copies/mL) at 4 weeks after inoculation (Fig. 1B), as reported recently by our group [23]. Six weeks after inoculation, three of six HBV- or HCV-infected mice were treated daily with 7000 IU/g/day of intramuscular IFN-alpha for two weeks. Treatment resulted in only 0.3 log decrease in mice serum HBV DNA level compared

to that in mice without treatment (Fig. 1A). In contrast, the same therapy resulted in a

rapid decrease in HCV RNA to undetectable levels as confirmed by quantitative PCR

(Fig 1B).

225   To investigate the direct interference of the two viruses, we performed double

infection experiments. Ten chimeric mice were first inoculated intravenously with 50

µL of HCV-positive human serum samples. Six weeks after HCV infection when the

mice developed HCV viremia, 50 µL of HBV-positive human serum samples were

inoculated intravenously in five of ten HCV-infected mice. All five mice became

230   positive for both HBV and HCV at two weeks after HBV infection. No significant

decrease in HCV RNA levels was observed in these superinfected mice before or after

the development of HBV viremia (Fig. 2A). After HBV infection, there was no

apparent decrease in HCV titer (Fig. 2B). Moreover, HBV DNA level in

HBV/HCV-coinfected mice was comparable with that of only HBV-infected mice

235   (Fig. 2B). These results suggest no interference between the two viruses in mice,

which lack immunocytes known to cause hepatitis.

To further investigate if infection with either of the two hepatitis viruses alters the

effect of IFN against the other virus, three HBV/HCV-coinfected mice were treated

with IFN-α (Fig. 3A). Such treatment resulted in a rapid decrease in HCV RNA in all

240   mice to undetectable levels as confirmed by quantitative PCR (Fig. 3B). In contrast,

no significant decrease in HBV DNA titers was observed in these mice (Fig. 3B).

These results are similar to the reduction of HCV RNA and HBV DNA in mice that

were infected with either of these hepatitis viruses. These results indicate that HCV is

more susceptible to IFN-α than HBV and that each virus does not alter the effect of

245 IFN on the other virus. Since the effect of IFN on HCV was not disturbed by HBV, we assumed that HBV has no effect on the signal from IFN receptor to IFN-stimulated genes. However, it is possible that HBV and HCV replicated in different cells in these mice. Since it was impossible to detect HCV protein and RNA in HCV-infected mouse liver by histological examination, we performed *in vitro* experiments.

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3.2 Production of both HBV and HCV producing cells and effect of IFN.

To investigate the effect of IFN on HBV and HCV *in vitro*, we created cell lines that produce both HBV and HCV. First, we established stably HBV-producing Huh7 cell lines. Three cell lines (Clone-39, -42 and -53) that produced HBsAg, HBeAg and 255 HBV DNA into the supernatant were selected (Table). These cell lines continuously produced HBV for more than three months (data not shown). Next, JFH1 RNA was transfected into these HBV-producing cell lines to produce both HBV DNA and HCV proteins into the supernatant. HBV DNA levels in the supernatants of these cell lines decreased in Clone-39, increased in Clone-42, and did not change in Clone-53 after 260 JFH1 transfection (Fig. 4A). On the other hand, HCV core antigen levels in the supernatants were higher in two of the three cell lines (Clone-39 and -42) than Huh7 and not different in the remaining cell line (Clone-53) (Fig. 4B). These results indicate that the production of each of the two viruses does not disturb the replication of the other virus.

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3.3 Effects of IFN on HBV and HCV in vitro.

The effects of IFN on virus production in both HBV and HCV-producing cell lines was examined by adding different amounts of IFN-α (0, 50 and 500 IU/mL) into the culture. The mRNA levels of intracellular IFN-stimulated genes such as MxA, OAS

270 and PKR increased in a dose-dependent manner in all three cell lines as well as parental Huh7 cells (Fig. 5A). Following the addition of IFN, no apparent reduction of HBV was noted in the supernatant of HBV/HCV-cotransfected cell lines (Fig. 5B). In contrast, the levels of HCV core antigen in the supernatant decreased in all three cell lines treated with IFN and the decrease was dose-dependent (Fig. 5C).

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4. Discussion

Although IFN treatment for chronic HCV infection has improved with the advent of PEG-IFN, the rate of viral eradication remains unsatisfactory [9]. The mechanism responsible for failure of IFN to eradicate the virus completely must be clarified. To

280 study the mechanism of viral resistance against IFN, analysis of viral interference may give us some hints because one of the major mechanisms of interference is through the action of IFN.

Accumulation of mononuclear cells is usually seen in the liver of infected individuals, in association with the state of inflammation. It is thus difficult to

285 examine the interference of hepatitis viruses in infection and replication in liver cells without taking into consideration the effect of these immune cells, as well as the chemokines and cytokines produced by these cells. Instead, the present study was designed to examine the interference between HBV and HCV in an experimental setup lacking such inflammatory interferences. The SCID-based human hepatocyte

290 chimeric mouse model is ideal for investigating such interaction. We expected a reduction of HCV after inoculation of HBV in HCV-infected mice, or failure to develop HBV viremia or low level HBV viremia in these mice due to viral interference. However, no reduction in HCV titers occurred in these mice and HBV infection developed in a manner similar to that in naïve mice (Fig. 2). We thus

295 confirmed that there is no interference between the two viruses in the absence of immune reaction via the infiltrating lymphocytes in the liver.

Wieland et al. reported that HBV did not induce any genes during entry an expansion in HBV-infected chimpanzee livers, and suggested that HBV was a stealth virus early in the infection [26]. Since no reduction in HCV was noted during and

300 after the development of high level HBV viremia, we assume that HBV escapes innate immunity via an excellent mechanism without evoking IFN production system in liver cells. Further study using double infected mice treated with anti-HBV nucleotide analogs and anti-HCV protease inhibitors should be conducted to confirm the present findings.

305 With regard to the use of IFN as a treatment, we initially assumed that HBV infection would prevent the effect of IFN on HCV and possibly vice versa in double-infection mice. Unexpectedly, the reduction of HCV by IFN therapy was quite similar in both mice infected with HCV only and those coinfected with HBV and HCV (Fig. 1, 3). This indicated that HBV does not disturb the effect of IFN through

310 signal transduction from IFN receptor through Jak-Stat pathway. However, it was considered possible that HBV and HCV infect different liver cells in mice and replicated without being affected by each other. It has been reported that the same

liver cell could be infected with both HBV and HCV [20, 27]. However, it was difficult in the present study to confirm that these two viruses replicate in the same

315 liver cell of mice because it is difficult to visualize HCV antigen and RNA in pathological sections of the mouse liver. To address this issue, we transfected HCV to stably HBV producing cell lines (Fig. 4). We thought that both HCV and HBV were produced from successfully HCV RNA transfected cells because transfected cells were stably HBV producing cells. Presence of the both hepatitis viruses in the same

320 hepatocytes has also been shown by a recent report by Bellecave et al. [20]. We showed in our cell line experiments the production of HBV from only HBV transfected cell line and that cells cotransfected with HBV and HCV did not show a clear effect of HCV replication on HBV production (Fig. 4A). Similarly, stable production of HBV did not alter the replication of HCV (Fig. 4B). These data are

325 consistent with a recent report [20] which showed that HCV could infect to cells producing HBV and suggest a lack of interference between the two viruses in liver cells.

Using HCV transfected HBV producing cell lines, we demonstrated that presence of HBV did not disturb the actions of IFN on HCV (Fig. 5C). HCV utilizes certain

330 machinery to disrupt the innate immune system. However, once exposed a large concentration of IFN, the virus shows high sensitivity as shown in the replicon system [16, 28]. Thus, HCV seems to have a relatively weak system to disturb the antiviral actions of IFN compared with HBV. In contrast, HBV showed strong resistance against IFN in cells with diminished HCV replication [29]. The fact that HBV does

335 not disturb IFN signaling but resists the actions of IFN suggests that HBV counteracts

the actions of IFN at IFN-induced anti-viral product levels.

Although the culture environment is different from the replicon system, the JFH1

strain seems relatively resistant to IFN [30]. This suggests that the core and envelope

proteins, which are absent in the replicon system, might play a role in IFN resistance.

340 However, we could not show any effect for HCV infection on the actions of IFN on

HBV replication. This finding suggests that the core and envelope protein have only a

weak effect on IFN resistance.

In clinical practice, HBV shows high resistance against IFN therapy. This is also

the case in the cell culture system, as we showed in this study and has been reported in

345 previous studies [20, 29]. The mechanism by which hepatitis viruses resist IFN need

to be clarified in order to develop new and effective therapies for eradication of these

viruses.

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FIGURE LEGENDS

Fig. 1. Changes in serum virus titers in mice inoculated with HBV- or HCV-positive human serum samples. (A) HBV DNA levels in six mice inoculated with HBV-positive serum samples. (B) HCV RNA levels in six mice inoculated with HCV-positive serum samples. Six weeks after inoculation, three of six mice were treated daily with (closed circles) or without (open circles) 7000 IU/g/day of IFN-α intramuscularly for 2 weeks. Mice serum samples were extracted every 2 weeks after inoculation. Data are mean ± SD ($n = 3$). The horizontal dashed line represents the detection limit (10^3 copies/mL).

Fig. 2. Comparison of HCV and HBV titers in experimentally infected mice. (A) Ten mice were inoculated with HCV-positive serum samples. Six weeks after HCV infection, 5 of the 10 mice were inoculated with HBV-positive human serum samples (closed circles). The remaining 5 mice (open circles) did not receive HBV inoculation. Data are mean ± SD ($n = 3$). (B) Serum HCV RNA titers in 5 mice infected with HCV before and at six weeks after HBV super-infection (left panel). Serum HBV DNA titers in 5 mice coinfected with HBV/HCV were compared with those of 5 mice with HBV infection only (Fig. 1) at 12 weeks after HCV inoculation (right panel). In these box-and-whisker plots, lines within the boxes represent the median values; the upper and lower lines of the boxes represent the 25th and 75th percentiles, respectively.

Fig. 3. Changes in serum HCV RNA and HBV DNA levels, and effects of IFN on HBV/HCV-coinfected mice. Three mice (mouse 1, 2 and 3) were inoculated with both

HBV- and HCV-positive human serum samples, and treated daily with 7000 IU/g/day

of IFN-alpha intramuscularly for 2 weeks. Mice serum samples were obtained every 2

weeks after injection, and HCV RNA (open circles) and HBV DNA (close circles)

465   were analyzed by quantitative PCR. The horizontal dashed line represents the

detectable limit (10^3 copies/mL) (A). Serum HCV RNA and HBV DNA titers in mice

before and after 2-week IFN-α treatment (B). In these box-and-whisker plots, lines

within the boxes represent median values; the upper and lower lines of the boxes

represent the 25th and 75th percentiles, respectively.

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Fig. 4. Virus titers in supernatants of HBV- or HCV-transfected cell lines. Huh 7 cells

were initially stably transfected with 1.4 genome length HBV DNA. Three cell lines

(Clone-39, -42 and -53) producing HBV DNA into the supernatant were selected. (A)

HBV DNA levels in supernatants of HBV-producing cell lines 72 hrs after

475   transfection with JFH-1 RNA (HCV+) or control plasmid (HCV-). (B) HCV core

antigen levels in the supernatant of parental Huh7 cells (Huh7) and HBV-producing

cell lines 72 hrs after transfection with JFH-1 RNA. Data are mean ± SD ($n = 3$).

Fig. 5. Effects of IFN treatment on HBV and HCV *in vitro*. Parental Huh7 cells

480   (Huh7) and three HBV transfected Huh7 cell lines (Clone-39, -42 and -53) were

transfected with JFH1 RNA. Immediately after JFH-1 transfection, the cell lines were

treated with IFN-alpha (0, 50 and 500 IU/mL) for 72 hrs. (A) Intracellular gene

expression levels of MxA, 2'-5' oligoadenylate synthetase (OAS), PKR were

measured. RNA levels were expressed relative to GAPDH mRNA. HBV DNA (B)

485 and HCV core antigen (C) in supernatants were measured. Data are mean ± SD ($n =$

3).

Table. HBV markers in supernatants of stably HBV-transfected cell lines.

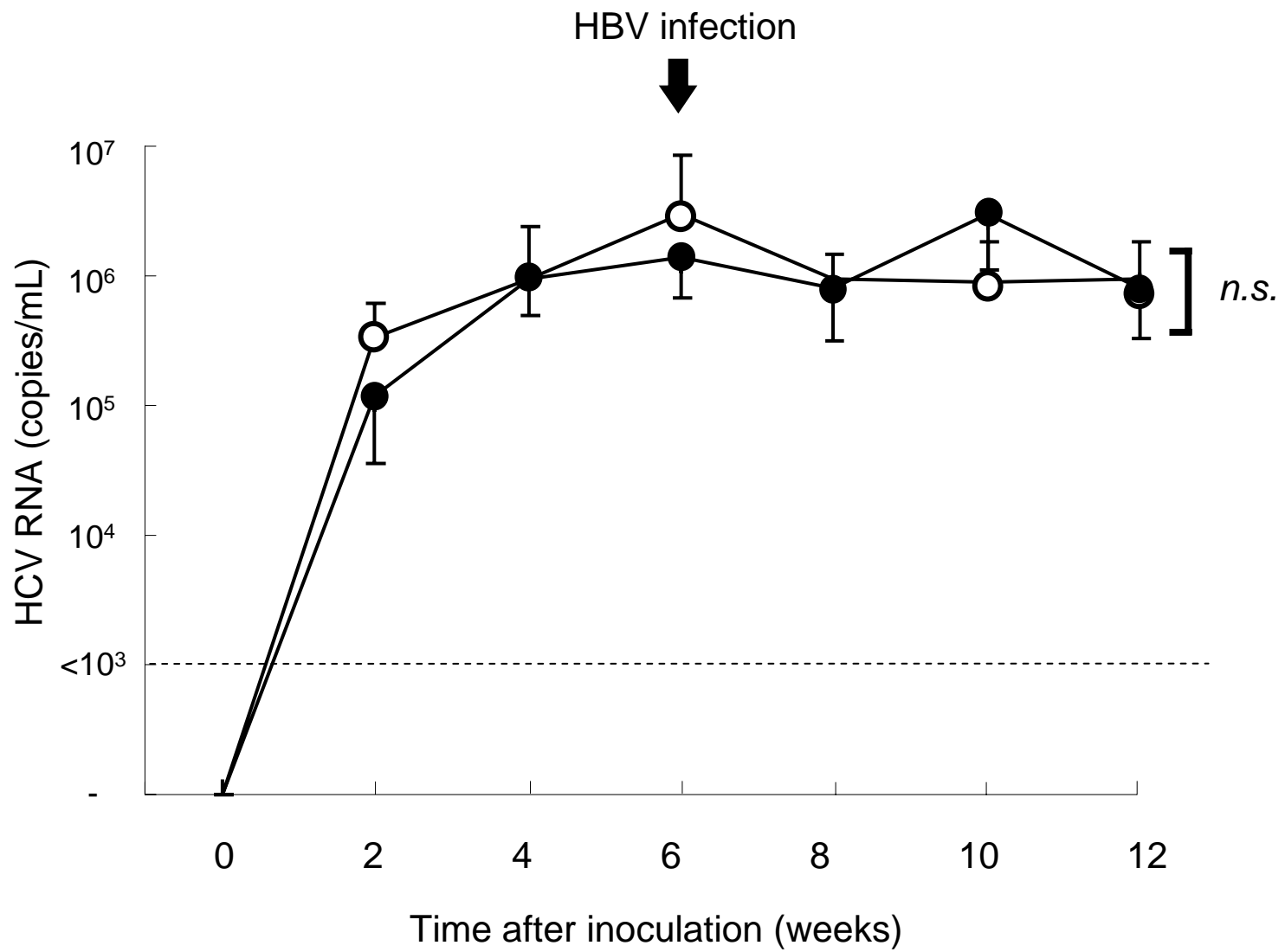| Clone	HBsAg (IU/L)	HBeAg (IU/L)	HBV DNA (log copies/mL)
39	0.46	4.57	5.2
42	8.16	1.34	5.3
53	0.08	9.29	5.4

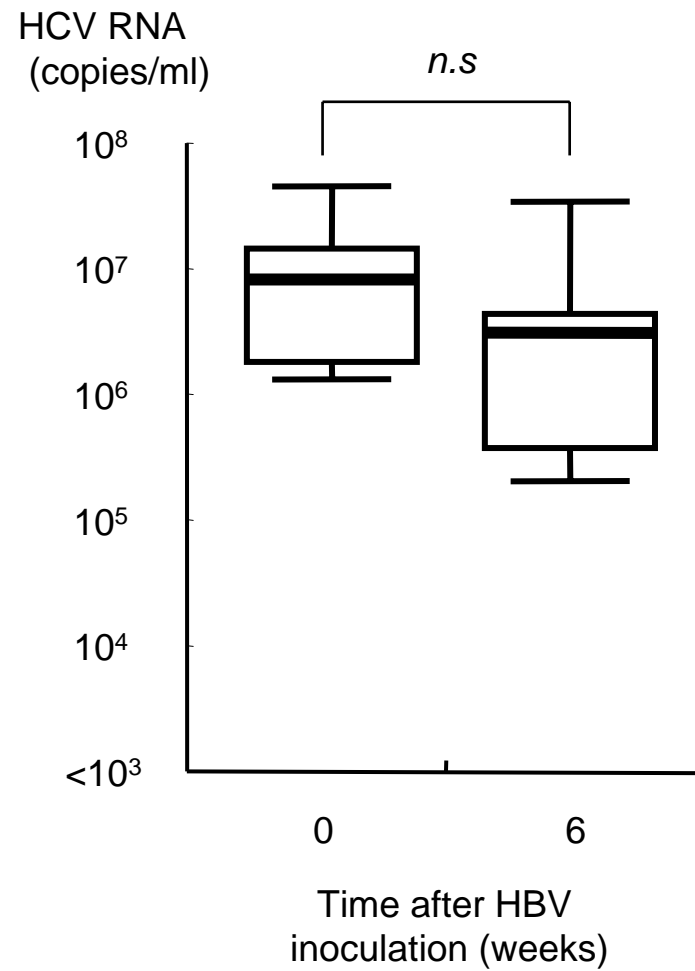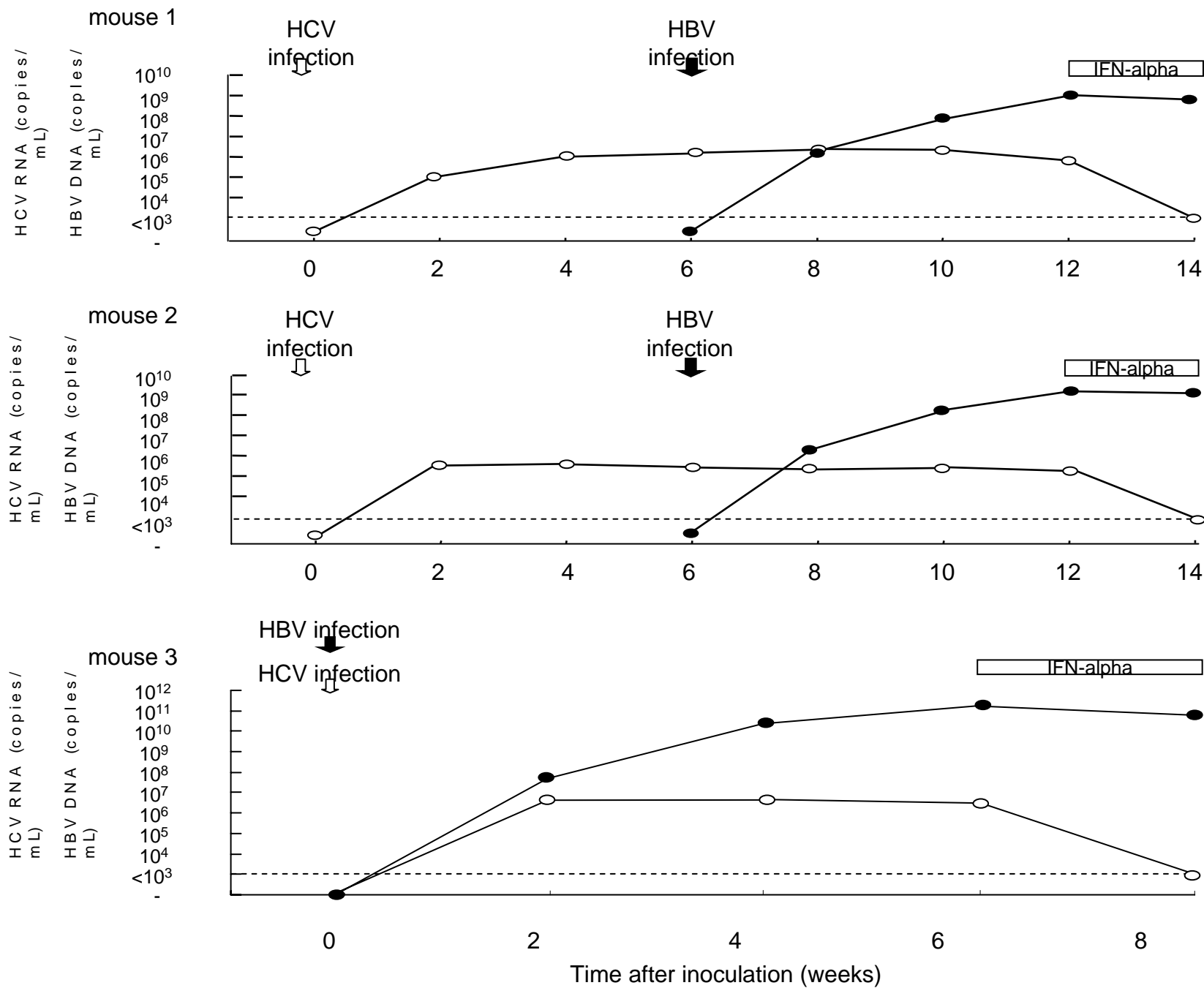490   HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen
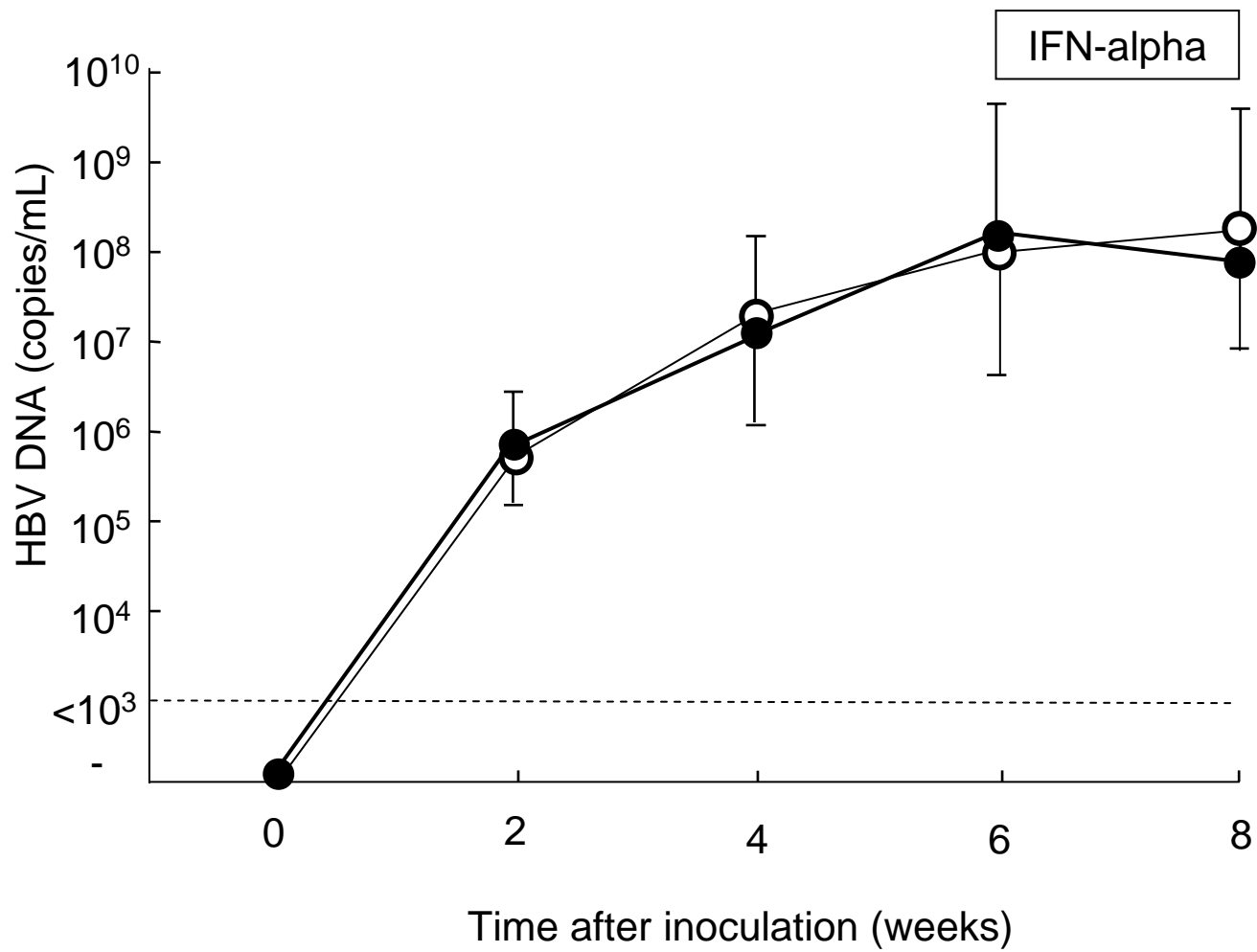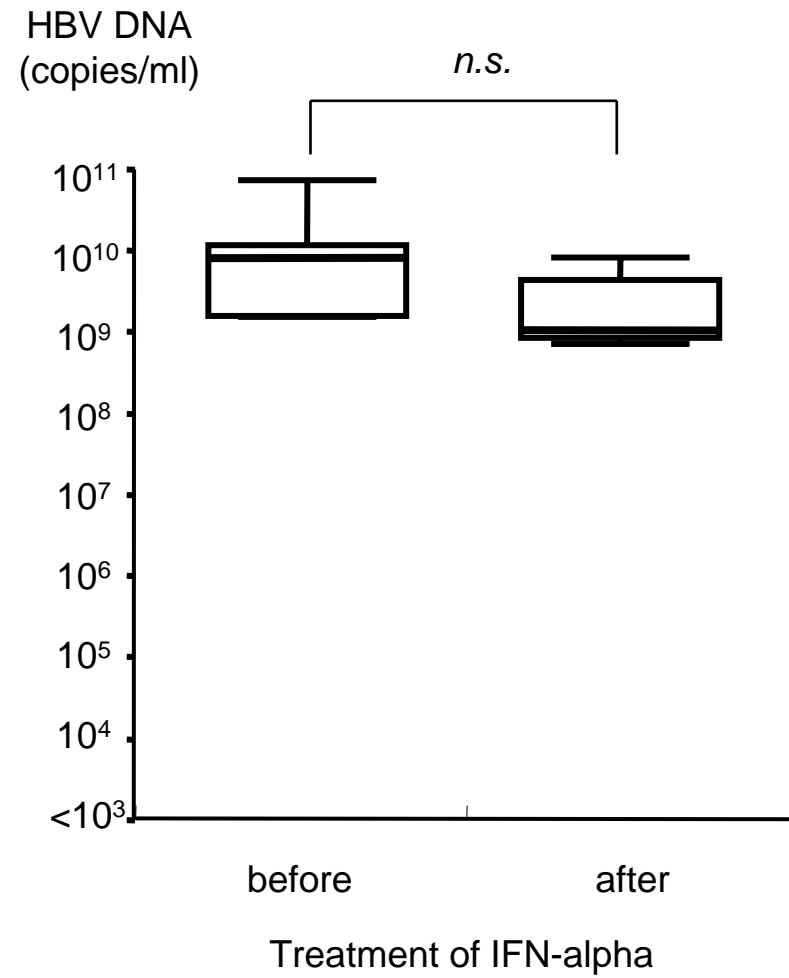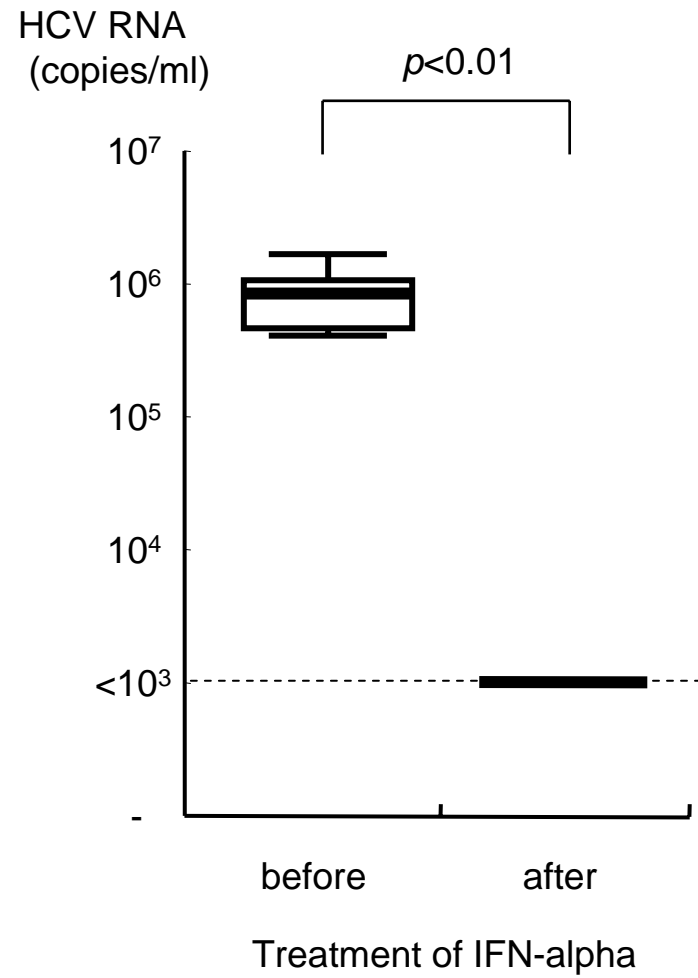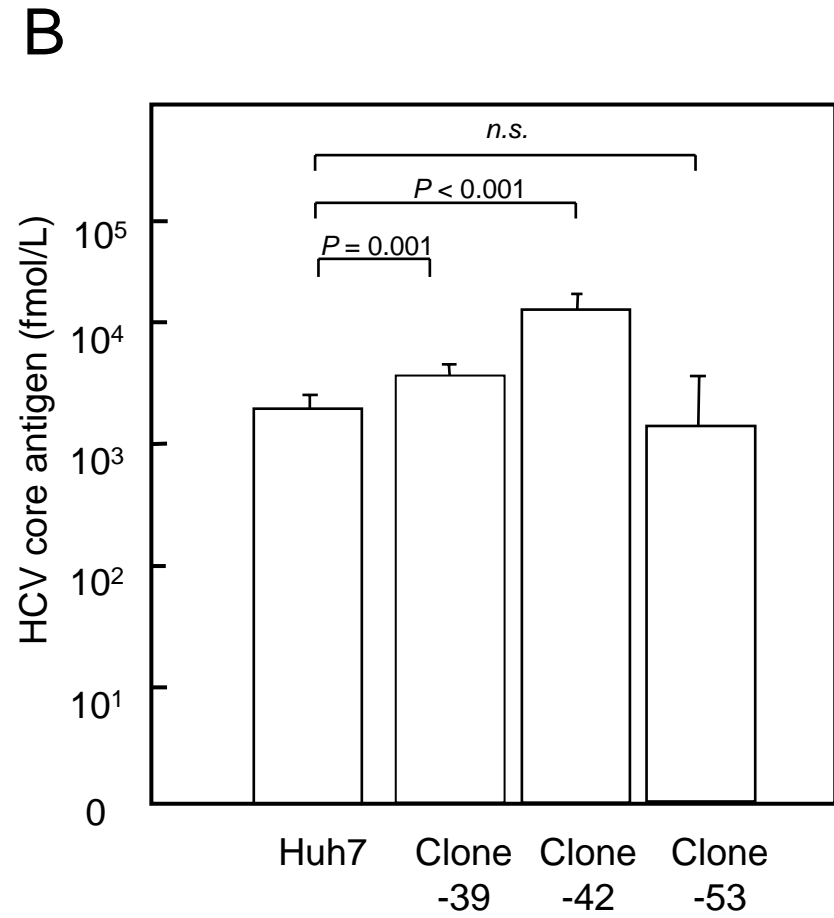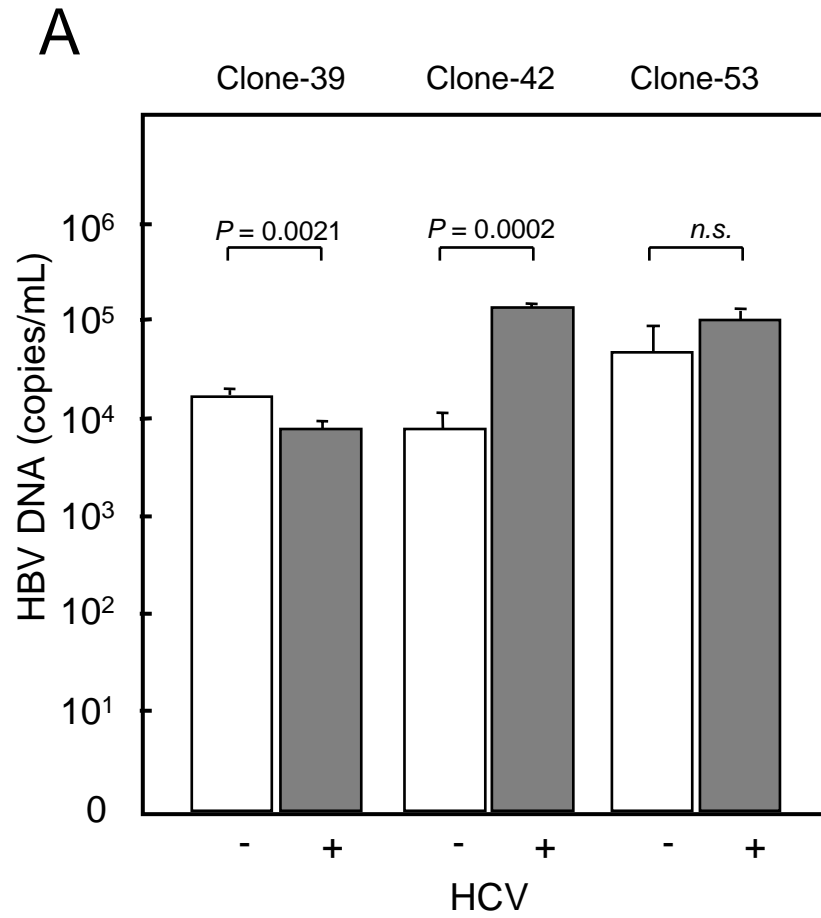
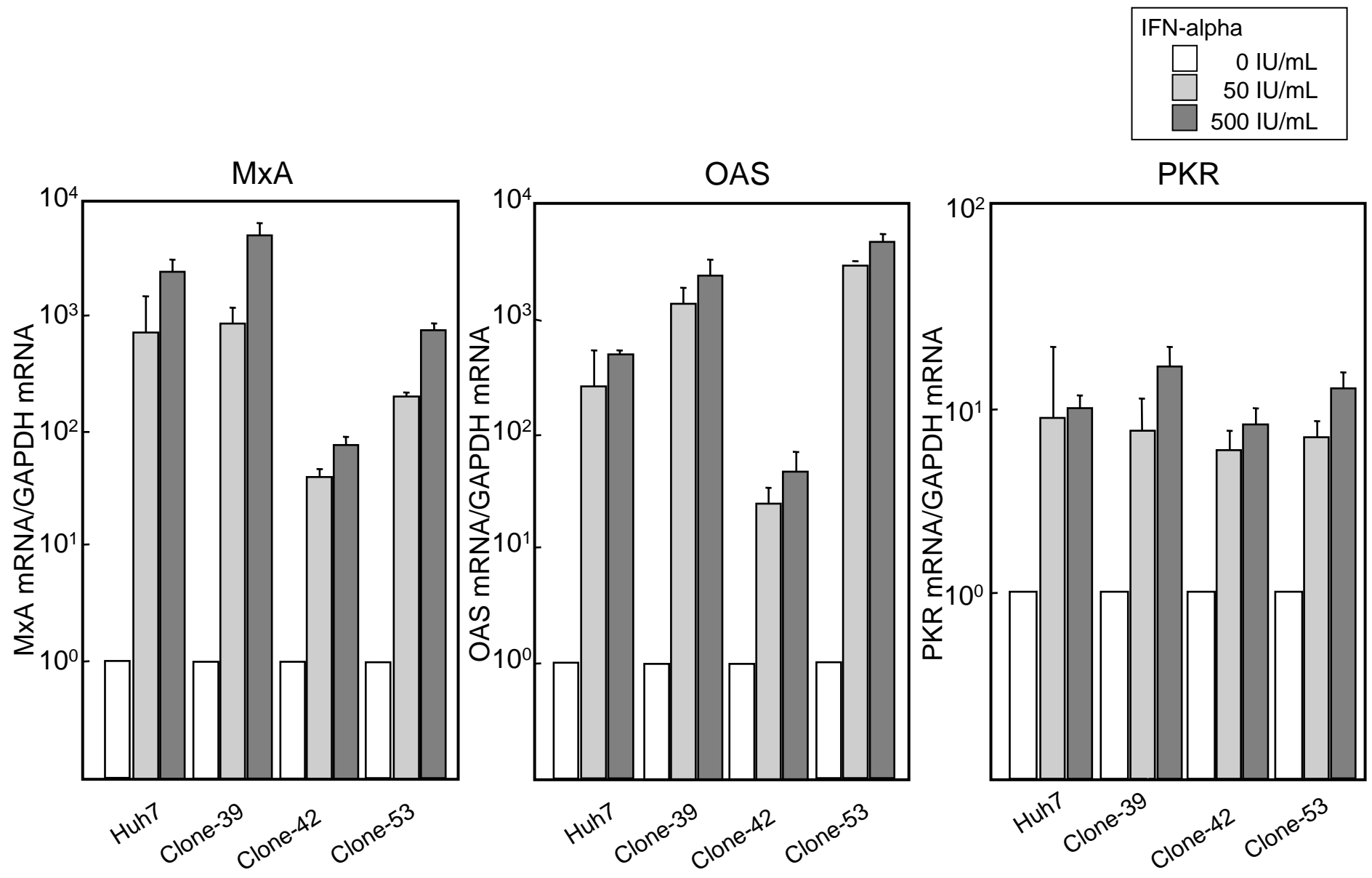Hiraga et al. Fig. 1A

IFN-alpha
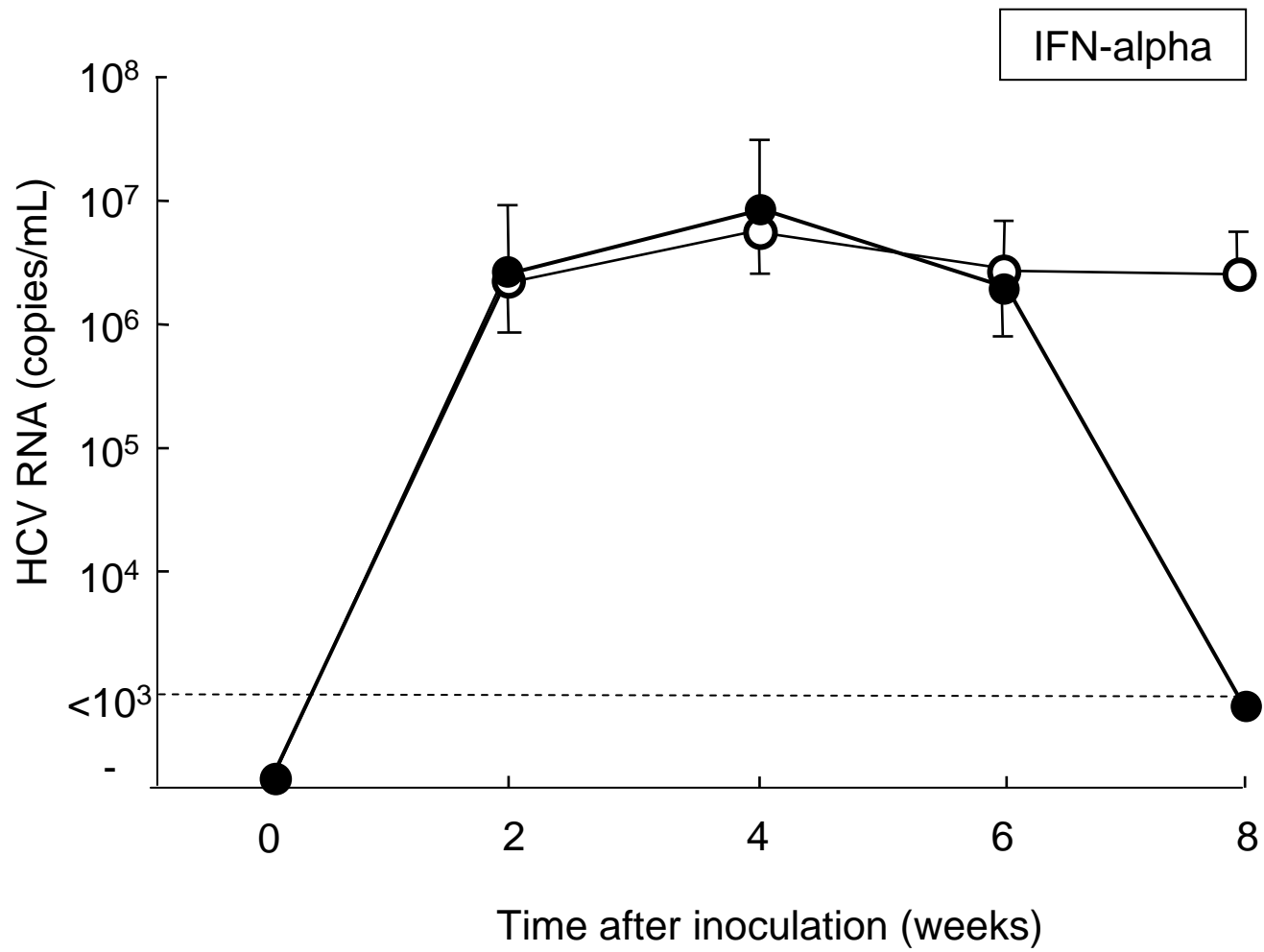
Hiraga et al. Fig. 1B

Hiraga et al. Fig. 2A
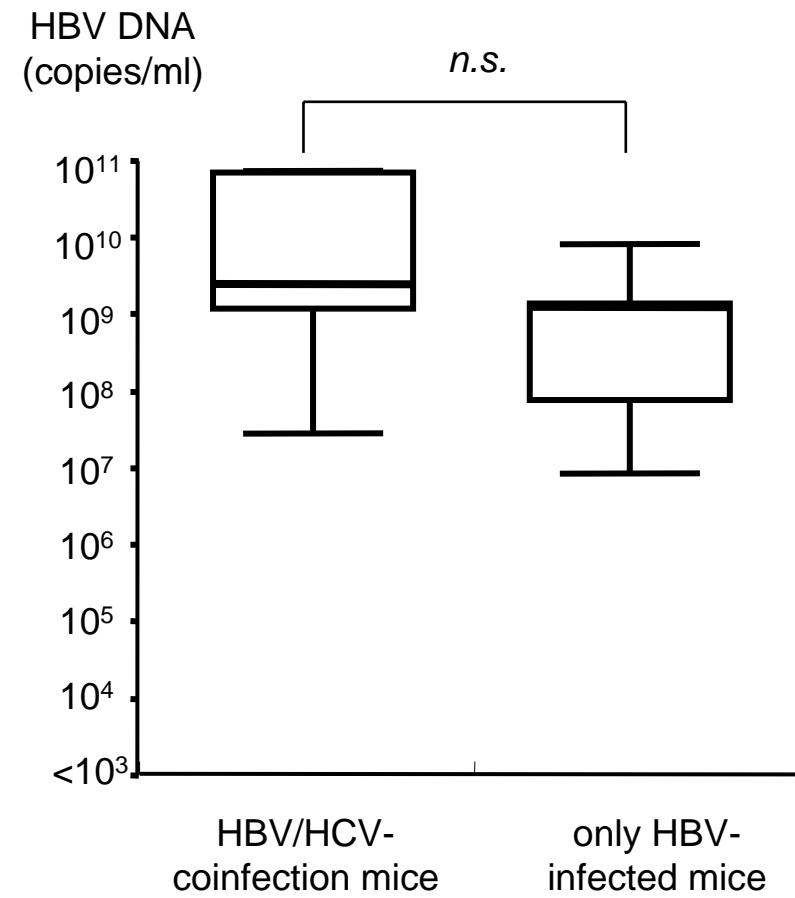
Hiraga et al. Fig. 2B
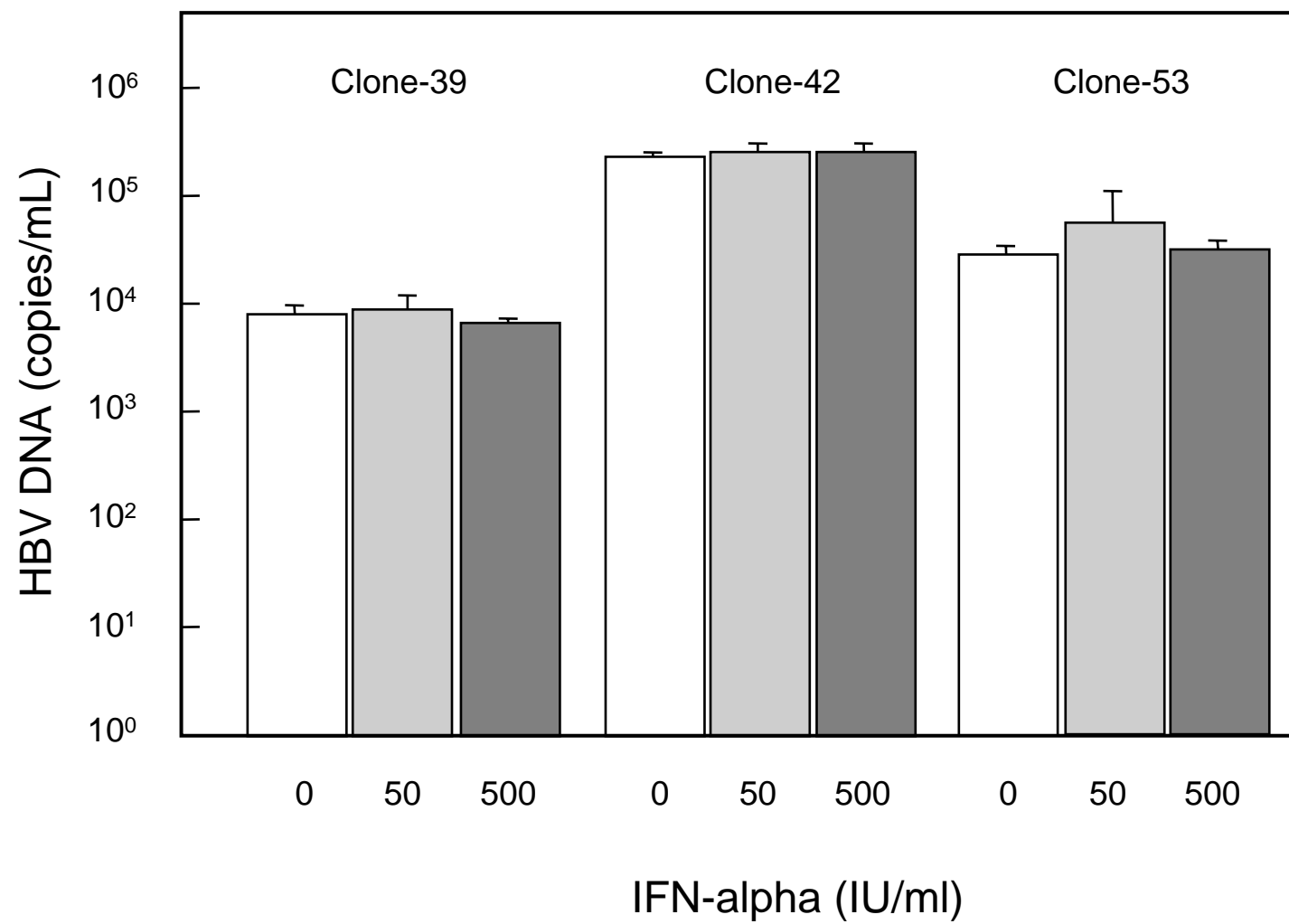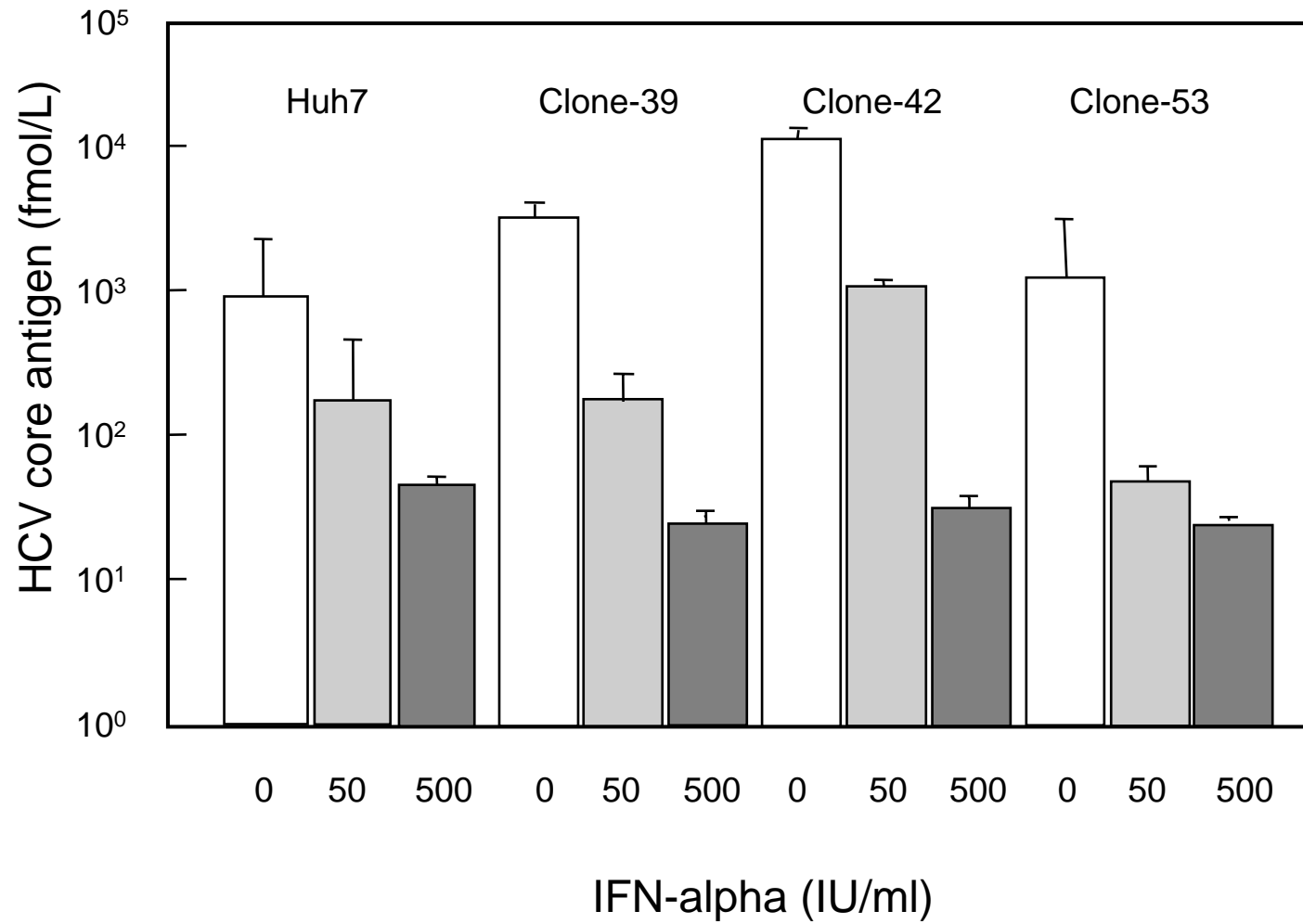
Hiraga et al. Fig. 3A

Hiraga et al. Fig. 3B

Hiraga et al. Fig. 4

Hiraga et al. Fig. 5A

Hiraga et al. Fig. 5B

Hiraga et al. Fig. 5C