Establishment of a Hepatitis B Virus Recombinant Baculovirus System and the Evaluation of its Function in Viral Antigen Production

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ABSTRACT

A handy *in vitro* viral replication system is mandatory for hepatitis B virus (HBV) study. A recombinant baculovirus with 1.3XHBV DNA construct was previously designed to infect HepG2 cells. We adapted this system and set up another one using 1.5XHBV DNA construct to generate our recombinant baculovirus, and we use Huh7 cells instead of HepG2 cells to establish this system. HBV genome was inserted into the baculovirus by recombination and the novel HBV recombinant baculovirus was identified by enzyme digestion. The viral stock was purified and its titre was determined. We then use the HBV recombinant baculovirus to infect Huh7 cell culture and demonstrate its ability to produce HBsAg. The production of HBsAg was first detected in the media three days after infection. Its production was in proportion to the loading amount of HBV recombinant baculovirus. A sustained HBsAg production could be achieved by superinfection of this recombinant virus to the already infected Huh7 cell culture. This system can be applied to the basic and clinical studies of HBV. We then introduced the recombinant baculovirus into the portal vein of the mice to test the in vivo model. The histologic examination of the mice showed the necroinflammatory changes. However, the typical groundglass pattern of HBV

infection found in human pathology was not demonstrated in the mice model. A microarray study was done to compare the RNA expression in the mouse liver infected with HBV-recombinant baculovirus and with baculovirus vector only for the future study.

Key words: hepatitis B virus, baculovirus

INTRODUCTION

The major obstacles in the study of HBV have been the inability of the virus to infect cells in vitro, and the lack of good animal model systems due to a relatively strict virus-host range. The chimpanzee system is very expensive and difficult to handle, and only available in very few centers in the world. The duck system and the woodchuck system are valuable models for studying HBV lifecycle, however, many significant differences still existed between animal hepadanviruses and human HBV. An easy-access HBV producing cell line is thus still of necessity for any new chemicals which might be effective in antiviral therapy for HBV.

Within the last decade, several HBV expressing cell line have been established. These cell lines were liver-derived human cell lines transfected with HBV DNA and the novel cell lines containing stably integrated HBV genomes were selected out (1). These cell lines did make contributions to the study of HBV biology and were useful for the trial of newly developed antiviral therapy (2,3). There are drawbacks for these cell lines, because they contained multiple copies of integrated HBV DNA and express HBV DNA with some strong heterologous promoters. This is not like the real situation. HBV DNA indeed

integrated into host genome, but this event is not required for the lifecycle of HBV. Besides, in human hepatocytes, the integrated HBV DNA is frequently rearranged and is often transcriptionally silent (4). The viral gene expression and replication is continuous in these cell line and impossible to be synchronized. Thus, it is impossible to precisely regulate the replication of HBV DNA at a specific time point. It is also impossible to superinfect the cell line to increase the dosage of HBV.

To overcome the above problems, an HBV recombinant baculovirus system was established (5). The previous study showed that by way of its endogenous promoter, a 1.3x genome of HBV construct inside a baculovirus could be successfully generated and transfected HepG2 cells. This HBV recombinant baculovirus-infected HepG2 cell line had advantages over the previous cell lines: (i) high-level of HBV expression. (ii) HBV replication level can be regulated over a wide range simply by changing the baculovirus multiplicity of infection (MOI). (iii) HBV replication in this system is detectable one day after infection and persisted for at least 11 days. (iv) The superinfection with this baculovirus can enhance or extend the infection period of the cell line. For these advantages, we decided to set up this novel system and adapt it to

test our new drugs and/or antiviral therapy in the future. In our study, we use a 1.5-unit length HBV DNA for generating the HBV expression baculovirus, and subsequently test in Huh7 cell line.

We then planned to use the HBV-recombinant baculovirus to infect human hepatocytes *in vitro* and rat hepatocytes *in vivo*. The RNA extracted from the human and rat hepatocytes after the HBV-recombinant baculovirus infection were subjected to microarray study (the strategy is listed below). Based upon the microarray results, we are likely to find out some possible candidate genes that may regulate the antiviral responses. It is also likely for us to find out possible viral receptor(s) in the mammalian hepatocytes. As the duck model system, it was shown the overexpression of gp180 (receptor) could enhance the duck hepatitis B virus entry into the hepatocytes (6). The microarray possibly will help us to find out overexpression of candidate receptor(s) genes when the recombinant HBV-baculovirus enters the hepatocytes, either *in vitro* or *in vivo* in the future.

MATERIALS AND METHODS

Cell culture. Sf21 insect cells (kindly provided by Prof. Bor-Leung Chiang) were maintained in complete TNM-FH medium (Grace's insect medium supplemented with 10% fetal bovine serum and 1% PS) in non-humidified incubator at 27℃ without CO_2.

Huh7 cells were maintained in Dulbecco's modified essential medium supplemented with 10% fetal bovine serum and 1% PS and were grown in humidified incubator at 37℃ and 5% CO_2. (All medium, serum, and antibiotic are from Gibco, Life Technology).

Construction of Baculovirus Transfer Vector. A recombinant transfer vector was constructed by excising a PvuII/PvuII fragment containing 1.5×HBV DNA (~5 kb) from pGEM3Z4.8 (kindly provided by Dr. Hui-Ling Wu) and cloned into the SmaI site of the multiple cloning region of pBlueBac4.5 (Invitrogen, Chatsworth, CA, USA). The insertion and orientation of the recombinant transfer vector were then confirmed by HindIII/KpnI enzyme digestion and DNA sequencing (Figs. 1 and 2).

Generation of Recombinant Baculovirus Containing 1.5× HBV DNA. Seed 2×10^6 Sf21 cells in a 60 mm dish, and gently wash twice with Grace's insect medium without FBS to remove the serum. Six μg of the purified recombinant transfer vector, 0.5μg of the Bac-N-Blue™ DNA (linear *AcMNPV* baculovirus DNA), and 20μl of Cellfectin® reagent were then co-transfected into the Sf21 cells according to the manufacturer's instructions (BAC-N-BLUE transfection kit, Invitrogen). After 4-hour of incubation at room temperature, 1 ml of complete TNM-FH medium was added. The dish was then

sealed with paraffin and incubated at 27℃ for 72 hours before harvesting the recombinant baculovirus.

Harvest the Recombinant Baculovirus by Plaque assay. Seed 5×10^6 Sf21 cells to 100 mm plate and infect the cells with 10^2, 10^3, and 10^4 dilutions of the transfection viral stock in TNM-FH medium at room temperature for one hour. The medium was then aspirated and an agarose/medium/X-gal mixture, consisting of 2.5 ml of 2.5% agarose solution (47℃), 2.5 ml of complete TNM-FH medium (47℃), and 5 ml of complete TNM-FH medium with X-gal (concentration: 150μg/ml, room temperature) was gently poured into the plate to overlay the virus-infected Sf21 cultured cells. The plates were sealed and incubated at 27℃ for 5 days until the distinguishable blue plaques are formed.

Select the Recombinant Baculovirus by PCR. The putative recombinant viruses were amplified from blue plaques. 72 hrs after infection, 0.75 ml of the medium was mixed with the same volume of 20%polyethylene/1M NaCl to precipitate the released virions from the blue plaques. The medium were then collected and labelled as P-1 stocks and store at 4℃. Viral DNA was purified by the standard Proteinase K digestion,

phenol/chloroform extraction, and isopropanol precipitation. The DNA was used as templates for polymerase chain reaction (PCR) (Primers P1: 5'-TCA CCA TAT TCT TGG GAA CAA GA-3' and P8: 5'-TTA GGG TTT AAA TGT ATG CCC-3') to detect if the virus isolates contain the 1.5× HBV DNA.

Preparation of High-Titer Viral Stocks. Seed 25 cm^2 flask with 2×10^6 log-phase Sf21 cells and add 20 μl of the P-1 viral stock in 5 ml complete TNM-FH medium. Incubate at 27℃ for 7 days until all the cells were lysed. Centrifuge at 1000g for 20 minutes to remove the cell debris and store at 4℃ as P-2 stock. Virus titres were determined by the plaque assay using 10^6, 10^7, and 10^8 dilutions of P-2 stock.

Infection of Huh7 cells with Recombinant Baculovirus Containing 1.5X HBV DNA. Seed 6-well culture plates with 10^5 Huh7 cells per well and the cells were grown for 16 to 24 hours before infection. On the day of infection, P-2 virus stock was diluted with DMEM/FBS/PS according to their titers to achieve the desired multiplicity of infection (moi) and adjust the final infection volume to1 ml. After 1 hour of infection at 37℃, the inoculum was aspirated and the cells were gently washed with HBSS (Gibco, Life Technology) for 3 times to remove all the virus-containing supernatant. 2.5 ml of

DMEM/FBS/PS was re-fed and maintained at 37℃ incubator.

Analysis of Secreted Hepatitis B Surface Antigen (HBsAg). Detection of HBsAg was performed by enzyme linked immunoassay kit (EIA, Abbott). The media from Huh7 cells were collected at several time points after infection and stored at -80℃ until analysis.

Animals and cannula insertion into portal vein. F-344 rats (160-200 gm) were fed a laboratory diet with water and food *ad libitum* until use and were kept under constant environmental conditions with a 12-hour light-dark cycle. The animals received humane care in compliance with the guidelines of the National Science Council (NSC 1997). Animals were maintained in separate cages. The cannula was an 18 cm length of polyethylene tubing (PE-50, ID 0.58 mm, OD 0.96 mm; Becton Dickinson, Sparks, MD). One end was sheathed with a 2.5 cm length of PE-160 tubing (ID 1.14 mm, OD 1.59 mm; Becton Dickinson, Sparks, MD). The two tubings were fused at one end with heat. The other end of the PE-50 cannula was beveled. A 3 mm section of a 20 GA BD Vialon catheter (ID 1.1 mm; Becton Dickinson, Sandy, Utah) was placed 7 mm from the tip of the bevel as a collar. The finished cannula was sterilized with ethylene oxide. A novel spring-guide-wire introducer needle was created by the modification of the spring-wire guide of the pediatric central venous catheterization set (0.46 mm x 45 cm, Arrow International, Inc). The 25 GA introducer needle was cut 6 cm from its point, and the cut edge was smoothed. This needle was adapted to the straight-tip end of the spring-wire

guide.

Rats were anesthetized intramuscularly with 75 mg/kg ketamine hydrochloride and 5 mg/kg xylazine. A midline laparotomy was performed, and the portal vein was exposed. The spring-guide-wire introducer needle was introduced through the cannula. Using this introducer needle, we punctured the portal vein about 1 mm down-stream from the stitch, advanced the needle 5 mm into the vein, and slid the cannula through the guide-wire into the portal vein up to the Vialon collar. The cannula was secured with a triple knot over the Violon collar with another triple knot just behind the collar. The guide-wire introducer needle was then withdrawn. After free blood returned into the cannula, the cannula was cleared with 0.1 ml heparinized saline (50 U/ml) and stopped with a blunt-ended 23 GA needle. Ampicillin (40 mg) and gentamicin (4 mg) were administered intraperitoneally. The abdominal wall incision was closed with 4-0 chromic surgical guts. The cannula was led through the subcutaneous tunnel to the interscapular incision (Fig. 3). The distal end of the cannula was anchored to the interscapula by suturing the cannula around the PE-160 tubing sheath to the skin. The patency of the cannula was reconfirmed and checked twice a week.

Histologic examination and RNA extraction from the animal livers. The rats were infused the recombinant virus through the portal cannula. In the meantime, the other rats infused with the wild type baculovirus served as the control group. Both the experimental group and the control group were sacrificed 72 hours after the infusion of virus. When sacrificed, (1) blood samples were collected through superior vena cava and sent for HBsAg assay; (2) a piece of liver tissue was sent for histologic examination;

the specimens were serially sectioned in 10-μm slices and stained with H&E to define the analyzed regions; (3) another piece of liver was homogenized with Trizol (Qiagen, Chatsworth, CA, USA) to extract RNA.

RESULT

Generation of the HBV Expressing Baculovirus

The 1.5X HBV DNA was excised from pGem3Z4.8, cloned into the baculovirus transfer vector pBlueBac4.5, and confirmed by restriction mapping (Fig. 1 and 2) and DNA sequencing. The recombinant baculovirus transfer vector and Bac-N-Blue^{TM} DNA, a modified linear baculovirus DNA, were then co-transfected into Sf21 cells. The recombinant baculovirus would produce β-galactosidase and yield blue plaques (Fig. 4). To confirm the successful construction of the recombinant baculovirus, a PCR with primers P1/P8 was designed to detect the presence of HBV DNA (Fig. 5) (8). The viral isolates were used for all subsequent viral amplification and titered by plaque assay.

Secretion of Hepatitis B Surface Antigen by infected Huh7 cells

We tested the ability of HBV recombinant baculoviruses to express HBV gene

products after infecting Huh7 cells. Huh7 cells were infected at different MOI and 1 ml of the medium was collected daily for analysis of HBsAg by EIA. At MOI=50, HBsAg was first detected 3 days after infection, and the titer of HBsAg increase daily during the 8-day period (Fig. 6). With different MOIs (= 0, 10, and 20), the production of HBsAg was basically dose-dependent (Fig. 7).

Superinfection of Infected Huh7 Cells with HBV Baculovirus

To determine whether the HBV expression in Huh7 cells could be bolstered by the recombinant baculovirus, we superinfected the already infected Huh7 cells 4 days after the initial infection at MOI=200. The media were collected daily for detection of HBsAg. The level of HBsAg titer increased after the second infection (Fig. 8).

Histologic finding of the HBV baculovirus-infected rat liver

The rat livers were process for H&E staining 72 hours after HBV baculovirus infusion through portal vein. We did not detect any ground glass pattern of human HBV infection. However, the necrosis of hepatocytes, the acidophilic bodies, and some hyaline membrane changes can be demonstrated (Fig. 9).

DISCUSSION

Baculovirus is a natural hepatotropic agent (9). It tends to predominantly infect the hepatocytes and won't replicate in mammalian cells (10,11). This nature renders this HBV recombinant baculovirus become a powerful *in vitro* HBV replication system. In this project, we aimed to set up an easy and quick system of HBV replication. Such a system will be very useful when we apply it to the study of the efficacy of antiviral therapies, the molecular and cellular profiles of viral-hepatocyte interaction, and even the hepatocytes responses to multiple viral infections.

We have now established the HBV recombinant baculovirus system. A 1.5X HBV genome was inserted into a baculovirus to construct this recombinant baculovirus. This system was well demonstrated to be successful in terms of the production of HBsAg, the dose-dependent manner of the HBsAg production, and the sustained production of HBsAg by a superinfection of the recombinant virus. We also know the temporal profile of HBsAg production in this system.

The next step is to prove the existence of all the replication intermediates of HBV in this system. If this can be done, the system can work as an *in vitro* HBV replication

system. In the future, this system can be applied to: (i) the evaluation of efficacy of antiviral therapies. (ii) The model for acute response to HBV infection in hepatocytes; we may check the molecular profiles of hepatocytes before and after infected by HBV recombinant baculovirus. (iii) The model of co-infection of HBV plus other viruses or superinfection of other viruses upon an HBV-infected hepatocyte cell lines.

By this study, we did demonstrate a successful establishment of HBV baculovirus system *in vitro*. The portal vein infusion of this recombinant virus into the rat liver is not as successful as the *in vitro* model. The failure to demonstrate ground glass pattern of human HBV infection in the rat liver may be due to: (i) different species with different manifestation of HBV infection (ii) failure of HBV baculovirus to enter the hepatocytes through the portal vein. We have to do other studies, like HBcAg immunohistochemical staining, HBV RNA expression, to prove the *in vivo* expression of HBV baculovirus. If all the above studies proved that this is not the right way for HBV baculovirus to express, it is likely that we should seek for some other animal models.

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LEGENDS OF THE FIGURES

Figure 1. HBV recombinant transfer vector, a PvuII/ PvuII fragments containing the entire 1.5X-genome length HBV construct was excised from pGem3Z4.8 and cloned into the baculovirus transfer vector pBlueBac4.5.

Figure 2. HBV recombinant transfer vector was confirmed by restriction mapping. The PvuII/ PvuII fragment containing the entire 1.5-genome length HBV construct is approximately 5 kb and pBlueBac4.5 is 4.9 kb. The linear size of the recombinant vector is approximately 10 kb. If this vector is cut with HindIII and KpnI simultaneously, the resulting 4.9 kb plus 5 kb will be difficult to separate.

Figure 3. Long-term portal cannulation in rats. The portal cannula was (A) inserted into the main portal vein, remaining secured onto the wall of portal vein by one stitch with a triple knot over the Vialon collar, and (B) was anchored in the interscapular area of the rat.

Figure 4. Successful recombinant baculovirus yield blue plaques

Figure 5. PCR result of recombinant baculovirus isolates. pGem3Z4.8 is used as the template of positive control.

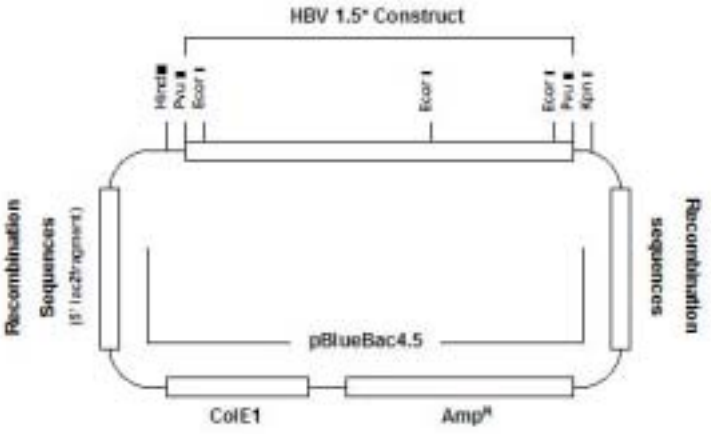
Figure 6. Analysis of HBsAg in the medium of Huh7 cells infected with HBV

baculovirus.

Figure 7. Analysis of HBsAg in the medium of Huh7 cells infected with different MOIs of HBV recombinant baculovirus.

Figure 8. The effect of superinfection of HBV recombinant baculovirus. The already infected Huh7 cells were superinfected with HBV recombinant baculovirus 4 days after the initial infection at MOI=200. The booster effect of HBsAg production could be seen.

Figure 9. The histological examination of the rat liver sacrificed 72 hours after HBV baculovirus infusion through the portal vein. (a) necrosis of hepatocytes (arrow) (b) an acidophilic body (arrow) showed an acute hepatitis reaction (c) perihapatocyte hyaline membrane changes, perhaps also due to the acute hepatitis reaction (d) the control rat liver.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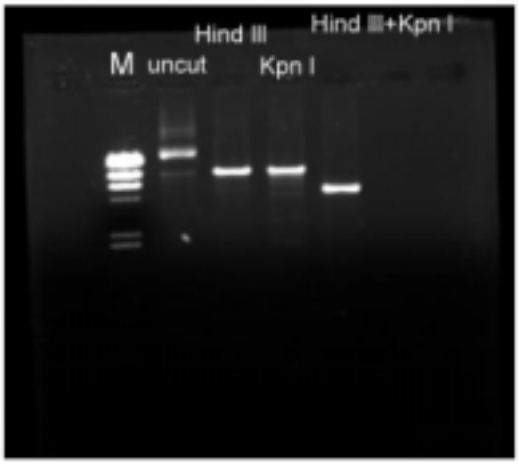
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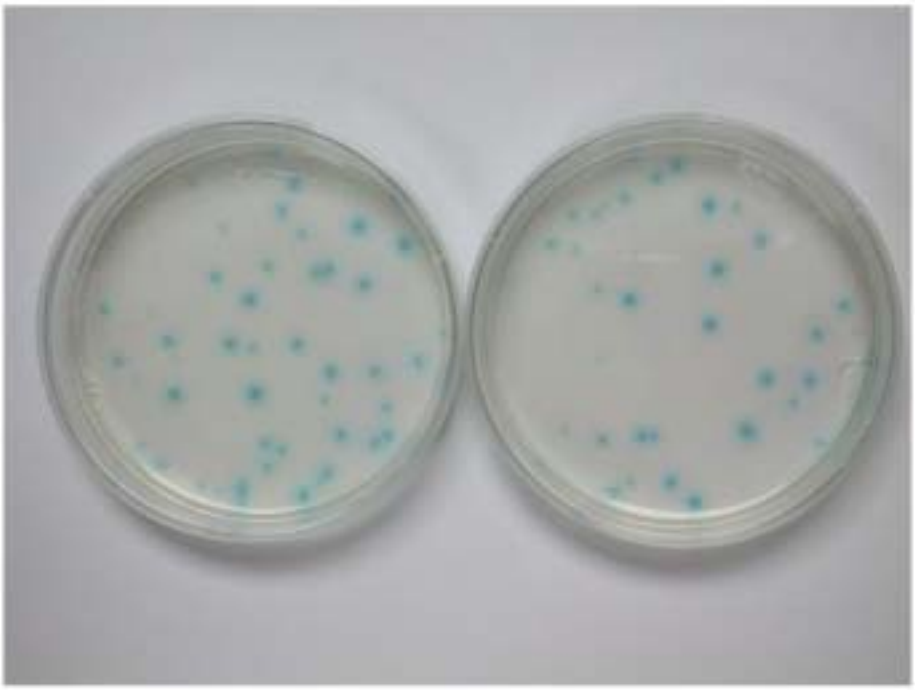

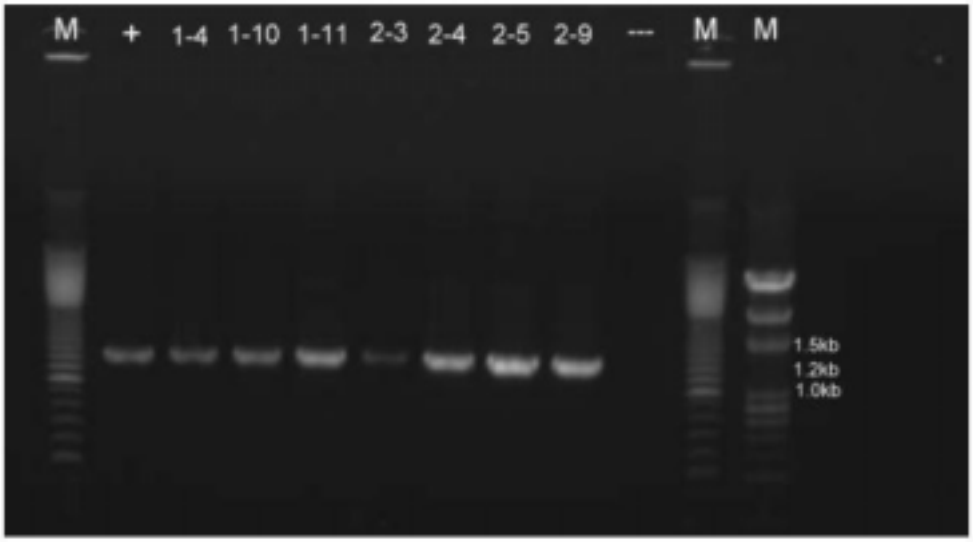
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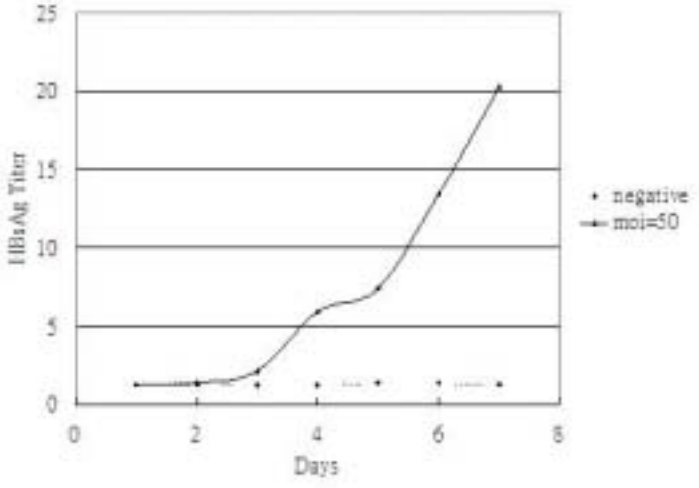
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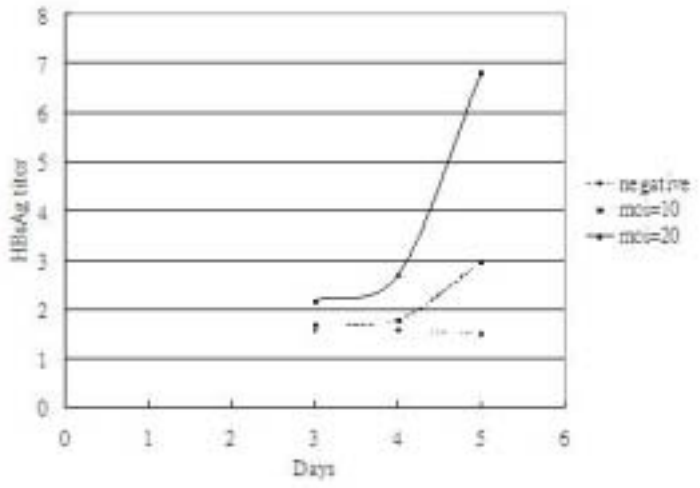
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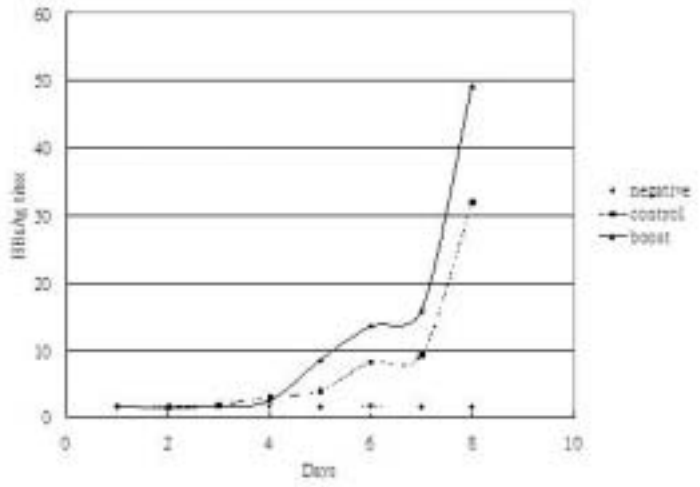
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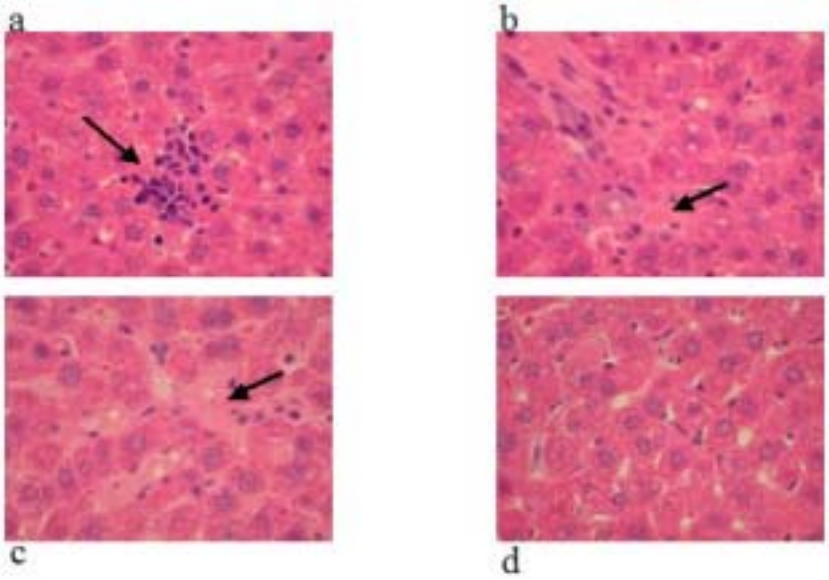
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