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Focus on hepatology in Japan

Hepatitis B virus (HBV)-transgenic mice as an investigative tool to study immunopathology during HBV infection

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Summary. An overview is given regarding the use of hepatitis B virus (HBV) transgenic mice as an animal model of the HBV-carrier state. Initially, we show how HBV-transgenic mice have contributed insights into the immunopathobiological processes during HBV infection and later, we show how this new information from the experiments with HBV-transgenic mice could be used to develop new methods to combat HBV infection.

By microinjecting the full or selected parts of the HBV-genome into the fertilized eggs of inbred mice, different laboratories have developed different lines of HBV-transgenic mice, which express products of the HBV genome and also show signs of HBV replication. Studies in HBV-transgenic mice have provided insights into the process of destruction of hepatocytes, the critical role of cytokines in controlling HBV replication and gene expression, mechanisms underlying the immune response defect in chronic HBV-carriers and the critical role of antigen presenting cells (APC), especially that of antigen presenting dendritic cells in persistent HBV infection. All this new information has given us a better understanding about HBV immunopathobiology, and has led to the development of new therapeutic approaches to combat HBV infection.

Keywords: Hepatitis B virus, transgenic mice, dendritic cells, immunobiology

The role of the immune system in liver diseases is not only complex but also poorly understood. This is specially true for infection with HBV, which is a noncytolytic envelop virus with a circular double-stranded DNA genome. A variety of disease processes are shown by HBV infection (Peters *et al.* 1991; Chisari 1995). Acquisition of HBV in adult life usually remains inapparent, and most acutely infected adults usually recover completely from the disease and clear the virus, although there are some cases of fulminant hepatitis following acute HBV infection. A small fraction, perhaps 5–10% of acutely infected adults become persistently infected with HBV and develop chronic liver diseases of varying severity. On the other hand, children born from HBV-carriers mothers usually become infected with HBV. This neonatally transmitted HBV infection is rarely cleared and more than 90% of such children become chronically infected with HBV. HBV is commonly spread from mother to newborn infants in highly populated areas of Africa and Asia, and there are several million people persistently infected with HBV at present. These chronic HBV-carriers develop varying degrees of chronic liver disease, which greatly increases their risk of developing more severe illnesses like liver cirrhosis and hepatocellular carcinoma (HCC). A large fraction of world's population suffer from and dies of these late complications of HBV

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infection, and this has been a constant burden on health care delivery systems throughout the world, especially in the developing countries.

It is generally thought that HBV is not directly cytopathic for the infected hepatocyte, which has been supported by the existence of large numbers of asymptomatic chronic HBV-carriers throughout the world carrying HBV but not showing any evidence of liver injury (Hoofnagle *et al*. 1987). Many interventional approaches have been developed to combat HBV infection, and interferon is widely used as an antiviral agent to fight against HBV (Periollo 1993). However, the low rate of efficacy of interferon (less than 30%), high price, profound side-effects, the parenteral route of administration and the incapability of interferon therapy to influence the progression to hepatocarcinogenesis has profoundly limited the use of interferon as a drug of choice for chronic HBV infection. The limitation of interferon therapy to successfully combat HBV infection in the majority of cases with chronic HBV is understandable because drugs like interferon, having mostly antiviral potentiality, have a limited role to fight persistent HBV infection. This is because the liver diseases caused by HBV are thought to be due to the immune response of the host to viral antigens, expressed by infected hepatocytes, and not due to the virus itself (Thomas *et al*. 1982; Chisari 1995). A great deal of evidence and experimental data strongly suggests that the magnitude of the cellular immune response is involved in disease pathogenesis, and the cellular and humoral arms of the immune response are required for viral clearance (Chisari 1995).

The proper understanding of the pathological processes, especially of the immunological mechanisms during chronic HBV infection is vital in order to develop a strategy for control of HBV infection, but it has been difficult to examine these immunological steps due to the limited host range of HBV and the lack of an *in vitro* system to propagate it. For this reason, until recently most studies of the immune pathology of HBV have been limited to the analyses of HBV-infected patients and chimpanzees, or of HBV-related hepadnavirus infections in woodchucks, ground squirrels, and pekin ducks. All these animal models are of outbred origin and the immune systems are not fully characterized.

Human HBV-carriers and characterization of the immune response

The pathogenic mechanisms responsible for the HBV-associated acute and chronic necroinflamatory liver diseases, and the viral and host determinants have only been partially defined until now. The immune response of the host to HBV-related antigens is important during the acquisition of HBV-carrier state, and also during the continuation of the persistent HBV-carrier state. The immune response of the host appears to be crucial in overcoming the HBV-carrier state because there is a rise in transaminase and evidence of hepatocyte damage before the clearance of virus, either spontaneously or by antiviral drugs (Peters *et al*. 1991). The destruction of hepatocytes in HBV-carriers is the manifestation of immune response of the host to HBV.

Several experiments have been done to understand the mechanism underlying the immune response defect in HBV-carriers. HBV-carriers have been reported to have a generalized immune response defect, such as decreased production of polyclonal immunoglobulins as well as specific antibodies (Dusheiko *et al*. 1983; Nowicki *et al*. 1986), delayed migration of lymphocytes and presence of suppressor lymphocytes (Brating & Berg 1983) and inhibitory effects of factors arising due to hepatitis (Yamauchi *et al*. 1988). Although the existence of a generalized immune response defect in HBV carriers has been shown by some, the most important problem lies in the defective immune response of HBV-carriers to HBV-related antigen, the most important of which is the circulating hepatitis B surface antigen (HBsAg) (Dusheiko *et al*. 1983; Fernan *et al*. 1989). The immune response to HBsAg is vital in HBV-carriers because on the one hand all HBV-carriers show high levels of circulating HBsAg, and on the other hand, the development of circulating anti-HBs is associated with the recovery from the chronic HBV-carrier state. Although HBV-carriers do not show an immune response to HBsAg, they respond to other HBV-related antigens, such as hepatitis Be antigen (HBeAg) and core antigen (HBcAg). The inability of HBV-carriers to adequately respond to HBsAg, in spite of the presence of high levels of circulating HBsAg, maybe due to the influence of the tolerogenic effect of HBsAg due to neonatal transmission of HBsAg from mother to neonates. Antigenic challenge during the neonatal period is considered as a tolerogenic rather than an immunogenic event (Burnet *et al*. 1950; Billingham *et al*. 1953). However, this does not explain why some adults become chronic HBV-carriers after acute infection with HBV during adult life. Moreover, recent studies have challenged the concept of neonatal tolerance and the immaturity of neonatal T cells (Ridge *et al*. 1996), and studies in HBV-carriers have shown the presence of HBsAg-specific lymphocytes and the ability of HBV-carriers to produce anti-HBs (Sylvan *et al*. 1985). It has been proposed that the inability to detect HBsAg-specific lymphocytes in HBV-carriers until now might be a matter of technical limitation only (Maruyama *et al*. 1992, 1993).

HBV-transgenic mice

With the advent of embryo microinjection technology, it was clear that many questions relating to HBV immunobiology and pathogenesis might be directly examined by introduction of a partial or complete copy of the HBV genome into transgenic mice. Several lines of HBV-transgenic mice have been prepared by introducing either the full HBV genome, or a partial or selected portion of the HBV genome into an inbred strain of mice (summarized in Table 1). The successful preparation of HBV-transgenic mice and the expression of HBV gene products have shown that mice might be a host for the expression of HBV-related products. Replication of HBV has also been documented in some lines of HBV-transgenic mice (Farza *et al.* 1988; Araki *et al.* 1989).

The hepatotropic nature of HBV gene expression has also been challenged in HBV-transgenics because HBV gene products have been shown in extrahepatic tissues such as testis, brain, gastrointestinal tract, and pancreas, etc. (Burk *et al.* 1988), that are unique to each transgenic lineage. This sporadic and unpredictable expression probably indicates the influences of integration sites, but, the supercoiled form of HBV DNA (cccDNA) has not been detected in any of these lineages. These studies have shown that HBV has the potential to be expressed and to replicate in cells other than hepatocytes, and HBV gene expression is developmentally regulated (Deloia *et al.* 1989).

Experiments in some lines of HBV-transgenic mice have shown that the expression of the HBV gene is positively regulated by androgens and glucocorticoids, providing an insight into the male predominance of HBV infection and increased viral burden with steroid therapy (Farza *et al.* 1987). These data, together with the relative liver specificity of HBV reflects multiple constraints at the levels of viral entry, replication and gene expression, and it also demonstrates that none of the constraints is absolutely specific for human hepatocytes.

HBV gene products in HBV-transgenic mice

HBV-transgenic mice were produced, in which the envelope coding regions were controlled either by native HBV regulatory elements, or in which the preS promoter was replaced by the inducible, liver-specific mouse metallothionein promoter or the constitutively active mouse albumin promoter to have more insights into the behaviour of different HBV gene products. By using these different constructs, it has been shown that the middle and small envelope proteins assemble into small 22-nm spherical particles that bud into the endoplasmic reticulum and are rapidly secreted by the cells (Chisari *et al.* 1986, 1987). In contrast, large HBV envelope proteins assemble into long, branching filamentous particles and become trapped into endoplasmic reticulum and are not secreted (Chisari *et al.* 1986, 1987). The progressive accumulation of these particles in the endoplasmic reticulum cause a dramatic expansion of endoplasmic reticulum in the hepatocytes, eventually causing ultrastructural and histological changes that are characteristic of ground glass hepatocytes found in the liver of chronically infected patients with integrated HBV DNA (Gerber *et al.* 1974a,b; Chisari *et al.* 1987).

In order to study the factors that influence the intracellular localization of nucleocapsid proteins and particles in the primary hepatocytes *in vivo*, transgenic mice that

Table 1. Hepatitis B virus transgenic mice

Gene Expressed	Characteristics	Reference
All HBV ORFs	Viral replication	Farza *et al.* (1987) Araki *et al.* (1989)
	Viral replication is not cytopathic	Guidotti *et al.* (1995)
	CTL-induced hepatitis	Guidotti *et al.* (1994b)
	CTL inhibit viral replication	Guidotti *et al.* (1994b)
Small S	Hormonal regulation	Farza *et al.* (1987)
	CTL and cytokine regulation of HBV gene expression	Gilles *et al.* (1992) Guidotti *et al.* (1994b, c)
	Small S is not cytopathic	Chisari *et al.* (1995)
Large and small S	Cytokine induced hepatitis	Gilles *et al.* (1992)
	CTL-induced hepatitis	Moriyama *et al.* (1990) Ando *et al.* (1993)
	HBsAg storage disease	Chisari *et al.* (1989)
X	HCC without prior liver disease	Kim *et al.* (1991)
	No HCC	Lee T-H *et al.* (1990)
Core	HBcAg is not cytopathic	Guidotti *et al.* (1994a)
	Core particles do not cross nuclear membrane	Guidotti *et al.* (1994a)
Precore	Precore is not cytopathic	Guidotti *et al.* (1995)

express the HBV core and precore proteins under the transcriptional control of liver-specific mouse major urinary protein were produced (Guidotti *et al.* 1994a). The precore protein was shown to be strictly secreted into blood as HBeAg and it is not detectable within any compartment of the hepatocyte by immunohistochemical techniques. The viral core protein, synthesized at a low rate by the hepatocytes in these animals was shown to be rapidly transported into the nucleus, where it accumulated and reached a critical concentration threshold for nucleocapsid particles to occur. The nucleocapsid particles remained in the nucleus until the nuclear membrane dissolved, whereupon they entered the cytoplasm. The cytoplasmic nuclueocapsid particles can not reenter the nucleus. Thus, the nuclear transport of nascent core monomers and dimers is quite brisk in the hepatocyte.

Role of HBV gene products in hepatocellular injury

One important fact coming out of all these studies is that most of the HBV gene products and the process of viral replication itself is not directly cytopathic to hepatocytes, at least at the levels attained in HBV-transgenic mice. Even overexpression of some of the HBV gene products, such as HBV core, precore, X, small and middle envelope proteins and viral replication have not been found to be cytopathic to hepatocytes (Gilles *et al.* 1992; Guilhot *et al.* 1993). A line of HBV transgenic mice (Araki *et al.* 1989), which expressed all HBV-gene products in sera and tissues and showed viral replication was followed for 24 months, but evidence of liver injury could not be found in these transgenics.

On the other hand, HBV large envelope protein has been found to compromise hepatocellular function and may kill the hepatocytes (Chisari *et al.* 1987). The large envelope protein has been found to accumulate in endoplasmic reticulum as filamentous particles and give the hepatocytes an appearance similar to ground glass hepatocytes found in chronic human HBV-carriers. The hepatocytes expressing high levels of large envelope protein have been found to be hypersensitive to some stimuli such as LPS, and IFN-γ. But, hepatocytes without excessive levels of large envelope proteins have been shown to be refractive to the effects of IFN-γ and LPS (Gilles *et al.* 1994). The mechanism underlying this observation and its clinical significance remains to be clarified.

Furthermore, prolonged storage of high concentrations of these large envelope proteins in the endoplasmic reticulum of ground glass hepatocytes (Chisari *et al.* 1987; Filippi *et al.* 1992) is directly cytotoxic to the hepatocytes, initiating a storage disease characterized by chronic hepatocellular necrosis and a secondary inflammatory and regenerative response that might lead to HCC (Chisari 1995). Thus, HBV-transgenic mice show that inappropriate expression of one gene product might be sufficient to set in motion a series of changes that might lead to malignant transformation. The incidence of HCC in this model corresponds to the frequency, severity and the age of onset of liver injury, which itself corresponds to the intrahepatic concentration of HBsAg and is influenced by the genetic background of the mice.

Experiments regarding core antigen expression in HBV-transgenic mice have shown that when HBcAg is detectable by immunohistochemical methods in the cytoplasm during HBV infection, it probably reflects either recent cell division with the release of HBcAg into the cytoplasm, or very high rates of core protein synthesis with de novo assembly of core particles in that compartment. This has been confirmed by the fact that antigenically active precore protein is so rapidly secreted that it is never detected in the cytoplasm, although steady state levels of HBeAg reach several micrograms per milliliter of serum (Guidotti *et al.* 1994a).

Cellular immune response in HBV transgenic mice and its significance

A pathological condition similar to acute hepatitis can be induced in HBV-transgenic mice by adaptive transfer of class I restricted, HBsAg-specific cytotoxic T lymphocytes (CTLs) (Moriyama *et al.* 1990). This indicates the possibility of an immune-mediated induction of hepatitis in human HBV-carriers. The severity of CTL-induced necroinflamatory liver disease has been shown to depend on the route of administration of CTLs, the production of IFN-γ by CTL, the number of HBsAg-specific hepatocytes in liver, the amount of HBsAg in each hepatocyte and the gender of the recipients (Ando *et al.* 1993). The sequence of events leading to hepatocyte damage has also been studied in HBV-transgenic mice. The HBsAg-specific CTLs after entering liver are attached to HBsAg-positive hepatocytes, triggering a signal which caused hepatocytes to undergo apoptosis (Ando *et al.* 1994a). This direct action of CTLs causes the classical feature of acute hepatitis with the appearance of scattered acidophilic Councilman bodies, characteristics of acute viral hepatitis in humans. Studies in HBV-transgenic mice have given clues to the relative contributions of direct and indirect effects of CTLs in hepatocellular damage. The hepatocyte destruction caused by the direct effect of CTLs has usually been limited to a few

hepatocytes because of effector and target cell ratio; this is usually very low in the livers of HBV-transgenic mice. The architectural constraints of the solid organ structure of the liver might be responsible for this, but other factors might be a player. These might be equally important in other examples of T cell-mediated cell destruction, although they are not widely appreciated because most of our knowledge regarding CTL mediated cell destruction is based on *in vitro* studies using much higher effector:target ratios, and cell mixtures in which free-ranging CTL movement is possible.

The indirect effect of CTLs has been shown to perform the bulk of hepatocyte damage in HBV-transgenic mice. Inflammatory cells are recruited in the vicinity of CTLs at about 4–12 h after injection, and cause the formation of necroinflamatory foci in which hepatocellular necrosis extends well beyond the CTLs. This indicates that most of the hepatocytes are killed by a mechanism other than by the direct killing effect of CTLs (Ando *et al*. 1993). A critical limiting point is found at this time. In most HBV-transgenics, the hepatocellular damage does not extend beyond this point, and this has a similarity with acute hepatitis in humans. The factors controlling this remain to be elucidated, but they may be extremely important regarding the pathogenesis of fulminant hepatitis. Again, similar to human acute hepatitis, only 5% hepatocytes are destroyed by this method. But, if the hepatocytes retain HBsAg, the destruction can proceed unlimitedly with a pathogenesis similar to fulminant hepatitis. If the disease process continues there is an accumulation of macrophages, Kupffer cells, and also hyperplasia of Kupffer cells which mimics HBV-induced fulminant hepatitis in man (Ando *et al*. 1993). All these findings in HBV-transgenic mice have shown that CTL-induced accumulation of necroinflammatory cells causes most of the hepatocellular destruction, not the CTLs themselves. This hypothesis has been proven because the occurrence of fulminant hepatitis can be reduced drastically by introducing neutralizing antibodies to γ-IFN, or by inactivation of macrophages by multiple injections of carrageenan.

Collectively, these observations suggest that most of histopathological damage in HBV-transgenic mice, except hepatocellular apoptosis, is mediated by antigen-nonspecific cytokines and by effector cells that have been activated by virus-specific T cells, but not by the CTLs themselves. The striking similarities between the immunopathological and histological features of HBV-transgenic mice and acute viral hepatitis in humans suggest that similar events probably contribute to the pathogenesis of human liver disease.

Inability of CTLs to enter most extrahepatic tissues in HBV-transgenic mice

HBsAg-specific CTLs are unable to destroy HBsAg-positive cells in other tissues when injected intravenously, although they can do so in hepatocytes (Ando *et al*. 1994b). However, injecting the same CTLs beneath the kidney capsule leads to destruction of HBsAg-positive renal tubules. This study give us an important clue about the microvascular barrier in these tissues over the easy accessibility of hepatic sinusoids. HBV has been reported to be stored in cells other than hepatocytes in humans, and many cells including cells of the haemapoetic origin have also been reported to be a reservoir of HBV (Kobra *et al*. 1986). The difficulty of HBsAg-specific CTLs to enter tissue other than liver also raises the problem of greater difficulty in clearing the extrahepatic reservoir of HBV in carriers, and this might be relevant to viral persistence.

Antiviral potential of immunomodulators

HBsAg-specific CTLs have been shown to destroy directly only about 5% of HBV-positive hepatocytes in HBV-transgenic mice. Further experiments have shown that soluble products of the immune response, especially IFN-γ, TNF-α and IL-2 have the capacity to suppress the steady state content of HBV messenger RNA in the hepatocytes of HBV-transgenic mice (Gilles *et al*. 1992; Guilhot *et al*. 1993; Guidotti *et al*. 1994b,c). These effects are conducted at a post-transcriptional level that selectively accelerates the degradation of HBV mRNA. The same events are found when CTLs secrete IFN-γ and TNF-α after antigen recognition. Now it has been shown that all viral gene products and replicative intermediates are destroyed by CTLs in a noncytolytic mechanism in HBV transgenic mice. Moreover, it has been shown that intrahepatic nucleocapsid particles and replicative intermediates are eliminated during hepatocellular regeneration after partial hepatectomy.

These observations suggest that in addition to destroying HBV-infected hepatocytes, a strong intrahepatic immune response to HBV might lead to suppress viral replication which may cure infected hepatocytes without killing them. Data suggest that there might be two pathways; one that degrades viral RNA in response to the release of IFN-γ and TNF-α from antigen-activated CTLs, and another that degrades nucleocapsid particles and their contents in regenerative hepatocytes.

Hepatocellular carcinoma in HBV-transgenic mice

As the HBX gene products show transcriptional transactivator properties and can transactivate cellular genes associated with cellular growth control, it has been suggested that unregulated expression of this gene may contribute to the malignant transformation of hepatocytes. The HBX protein has also been shown to inhibit p53 function *in vitro*, suggesting another role of this viral transactivator in HCC.

Although, some have reported HCC in HBX-transgenic mice (Kim *et al*. 1991), others have not observed such development (Lee *et al*. 1990). The difference in mouse strain may have played a role in this respect. CD1 mice develop spontaneous HCC and HBV-transgenic mice developed on a CD1 background developed HCC in most of the studies (Kim *et al*. 1991). They showed that the X protein may act as a cofactor for malignant transformation. The genesis of HCC in HBV-transgenics might be partly due to swelling of endoplasmic reticulum, resulting in spontaneous death of hepatocytes leading to a condition of secondary inflammation and regeneration. In addition, production of oxygen radicals is increased and antioxidants such as glutathonine and catalase are greatly decreased in the liver of susceptible HBV-transgenics. These changes are usually associated with a dramatic increase in oxidative damage of DNA. These events may lead to random DNA mutation and carcinogenesis. Although HBX gene products have been shown to be related to hepatocarcinogenesis, it should be borne in mind that HCC generation in HBX-transgenic mice occurs without hepatitis. However, HCC develops in humans usually after hepatitis due to both HBV and hepatitis C virus, and always after several bouts of hepatitis (Dunsfold *et al*. 1990; Peters *et al*. 1991).

HBV transgenic mice as an investigative tool to have insight into the immune response during chronic HBV infection

The defective immune response in HBV-carriers has been explained from the viewpoints of immunogenic tolerance of HBV-carriers to HBsAg arising from the antigenic challenge with HBsAg during neonatal period (Billingham *et al*. 1953), defective functions of T and B lymphocytes (Fernan *et al*. 1989), production of inhibitory factors (Brating & Berg 1983), and defective production of interferon (Onji *et al*. 1989). However, corroborative studies could not be performed in human HBV-carriers due to the limitation of performing mixed culture experiments due to MHC mismatching among individuals, the inability to perform experiments with exogenous antigens in humans, and limitations in isolating different cell populations from humans including difficulty in isolating antigen presenting cells. HBV-transgenic mice have been found to be a suitable animal model for the HBV-carrier state because HBV-transgenic mice harbour HBV from the neonatal period, and express HBV-related antigens in sera and liver throughout their life (Araki *et al*. 1989). The abnormal immune response in human HBV-carriers is attributed to both HBV and to liver injury (Brating & Berg 1983). HBV-transgenic mice are a particularly suitable model for the study of immunopathogenesis during HBV infection, because HBV-transgenic mice do not show liver injury and thus the role of liver injury is minimized.

HBV-transgenic mice expressing all HBV-related mRNAs and antigens in tissue and sera and showing no evidence of liver injury were used to characterize the immune response defect in HBV-carriers (Araki *et al*. 1989). HBsAg-specific lymphocytes could not be detected in these HBV-transgenic mice *in situ*, and injection of HBsAg in complete Freund's adjuvant (CFA) did not induce the production of anti-HBs or HBsAg-specific lymphocytes (Akbar *et al*. 1993). The HBsAg is a completely T-cell-dependent antigen and the immune response defect of HBV-transgenic mice to HBsAg might be a feature unique to HBsAg, but it might also represent a very specific defect of immune regulation. In order to answer this, we looked at the immune response of HBV-transgenic mice to keyhole limpet haemocyanin (KLH), a HBV-unrelated, T-cell-dependent antigen to clarify whether the inability to respond to HBsAg is due to immunogenic tolerance or due to a part of more elaborative complex response defect (Akbar *et al*. 1993). HBV-transgenic mice were immunized with two intraperitoneal injections of KLH (5 μg) in phosphate buffered saline, separated by 2 weeks, and the production of anti-KLH Ab and proliferation of KLH-specific T cells was analysed, 2 weeks after the second immunization and compared with the KLH-specific immune response in normal C57BL/6 mice, the original haplotype on which the HBV-transgenic mice were prepared. Only 6 of 18 (33%) of the HBV-transgenic mice, injected with KLH produced very low levels of anti-KLH Ab, whereas the rest did not produce any anti-KLH Ab in sera. On the other hand, all ($n = 30$) age, sex and histocompatibility-matched normal C57BL/6 mice produced significantly higher levels of anti-KLH Ab after being immunized with the same amounts of KLH. Moreover, lymphocytes from only 3 of 14 KLH-injected HBV-transgenic mice showed a very low degree of proliferation *in vitro* in response to KLH in culture, whereas, all ($n = 16$) KLH-primed normal

C57BL/6 showed significantly higher levels of KLH-specific proliferation. Thus, experiments using KLH as an antigen, have found that the inability of HBV-transgenic mice to respond to HBsAg is not completely attributable to the induction of tolerance to HBsAg arising from antigenic challenge during neonatal period, so HBV-transgenic mice were predicted to have a defective immune response to other T-cell-dependent antigens. The mechanism underlying the low production of anti-KLH Ab and insignificant proliferation of KLH-specific lymphocytes in HBV-transgenic mice was investigated using *in vitro* experimental systems. The defective immune response of HBV-transgenic mice to KLH could not be mainly attributable to a defective function of lymphocytes because the production of polyclonal IgM and IgG by lymphocytes and the proliferation of T cells and of B cells in response to concanavalin A and LPS were not significantly different between HBV-transgenics and normal C57BL/6. A series of experiments (Akbar *et al*. 1993, 1996) showed that the defective response of HBV-transgenic mice to KLH was not attributable to a defect of lymphocytes, but was due to a defective function of antigen presenting cells (APCs). Macrophages and dendritic cells are the two most potent APCs (Steinman 1991), so experiments were carried out to clarify the role of respective APC populations in regard to the immune response defect to T-cell-dependent antigen in HBV-transgenic mice. Positive selection experiments in which pure populations of dendritic cells and macrophages were added, and negative selection experiments in which dendritic cell populations were depleted by using antidendritic cell antibody and complement showed that the defective role of antigen presenting dendritic cells but not of macrophages was primarily responsible for the immune response defect of HBV-transgenic mice in response to T-cell-dependent antigen (Akbar *et al*. 1993). The availability of HBV-transgenic mice has made it possible to characterize the specific cell population responsible for the defective immune response to specific antigens since it has been possible to isolate dendritic cells from the murine system; furthermore there are specific immunological markers of murine dendritic cells. However, experiments with human dendritic cells are still very difficult due to the inability to isolate populations of fresh human dendritic cells from peripheral blood, and due to a lack of specific markers of human dendritic cells.

Overcoming the immune response defect in HBV-transgenic mice in coculture experiments

We speculated that if the defective function of dendritic cells is primarily responsible for the failure of HBV-transgenics to respond to T-cell-dependent antigen, then this defect could be challenged *in vitro* using dendritic cells from syngeneic mice in mixed cultures. A series of coculture experiments *in vitro* showed that although lymphocytes from KLH-primed HBV-transgenic mice did not produce anti-KLH Ab *in vitro* when cultured with KLH and dendritic cells from HBV-transgenic mice, the same lymphocytes produced anti-KLH Ab when cultured with dendritic cells from syngeneic normal mice or dendritic cells from HBV-transgenic mice, treated with γ-IFN, known to upregulate the APC function of dendritic cells (Akbar *et al*. 1996). The mechanism underlying the defective function of dendritic cells in HBV-transgenic mice was found to be due to a low stimulatory capacity of dendritic cells in allogeneic MLR and in oxidative mitogenesis, and further studies showed significantly lower levels of MHC class II on dendritic cells from HBV-transgenic mice compared with normal C57BL/6 mice (Akbar *et al*. 1993).

Production of HBsAg-specific antibody by HBV-transgenic mice

Although a defective function of antigen presenting dendritic cells was seen in HBV-transgenic mice to response to KLH, we were not sure whether the defective function of dendritic cells in HBV-transgenic mice really contributed to the immune response defect to HBsAg. To have more insight in this regard, HBV-transgenic mice were injected with 2 injections of HBsAg in CFA at an interval of 4 weeks and sacrificed 2 weeks after the last injection. Anti-HBs or HBsAg-specific lymphocytes could not be detected when spleen cells from HBsAg-injected HBV-transgenic mice were cultured with dendritic cells from HBV-transgenic mice. But, when lymphocytes from HBsAg-injected HBV-transgenic mice were cultured with dendritic cells from normal C57BL/6 mice or with dendritic cells from HBV-transgenic mice, treated with γ-IFN, there was production of anti-HBs *in vitro* in all cultures (Kurose *et al*. 1997). Similarly, HBsAg-specific proliferation of lymphocytes from HBsAg-injected HBV-transgenic mice could not be achieved in cultures containing lymphocytes from HBsAg-injected HBV-transgenic mice with dendritic cells from syngeneic HBV-transgenic mice. But, lymphocytes from HBsAg-injected HBV-transgenic mice responded to HBsAg when cultured with dendritic cells from normal C57BL/6 mice (Table 2). These two series of experiments in HBV-transgenic mice showed how the inability of murine HBV-carriers to respond to HBsAg or KLH was related to the function of the antigen presenting dendritic cells.

Table 2. Critical role of dendritic cells for anti-HBs production in HBV-transgenic mice

T/B cells	APC	DC	Mac	Anti-HBs
NM (i)	NM			4.14 ± 0.90
TM(i)	TM			0.65 ± 0.20
TM(i)		NM		4.35 ± 0.67
TM(i)	NM + 33D1 + C			0.89 ± 0.25
TM(i)			NM	0.70 ± 0.20

T/B cells were collected from HBV-transgenic mice (TM) or normal C57BL/6 mice (NM), injected twice with HBsAg in CFA (i) and cultured with antigen presenting cells (APC), dendritic cells (DC) or macrophages (Mac) for 10 days with HBsAg in culture and levels of anti-HBs was measured in culture supernatants by an ELISA. Results were shown as levels of anti-HBs in cut-off index, more than 1.0 indicated positive anti-HBs response. 33D1 is a dendritic cell-specific antibody and depletes dendritic cells in the presence of complement (C).

Revisiting the concept of neonatal tolerance to HBsAg in HBV-carriers

Most of the children who are born from HBV-carrier mothers usually become HBV-carriers, and this has been explained in terms of neonatal tolerance, the basic concept of which originated from the work of Burnet *et al.* (1950), who showed that antigenic challenge during neonatal period is a tolerogenic event rather than an immunogenic one. HBsAg is seen in HBV-transgenic mice from 17 days of gestation and high levels of HBsAg are seen in HBV-transgenic mice after birth in sera and high levels of HBsAg are constantly expressed in sera throughout life. The capacity of these transgenic mice to produce anti-HBs *in vitro* in cultures containing dendritic cells from normal C57BL/6 mice, and the proliferation of HBsAg-specific T lymphocytes from HBsAg-injected HBV-transgenics in the presence of dendritic cells from normal C57BL/6 mice led us to conclude that the circulating HBsAg in HBV-transgenic

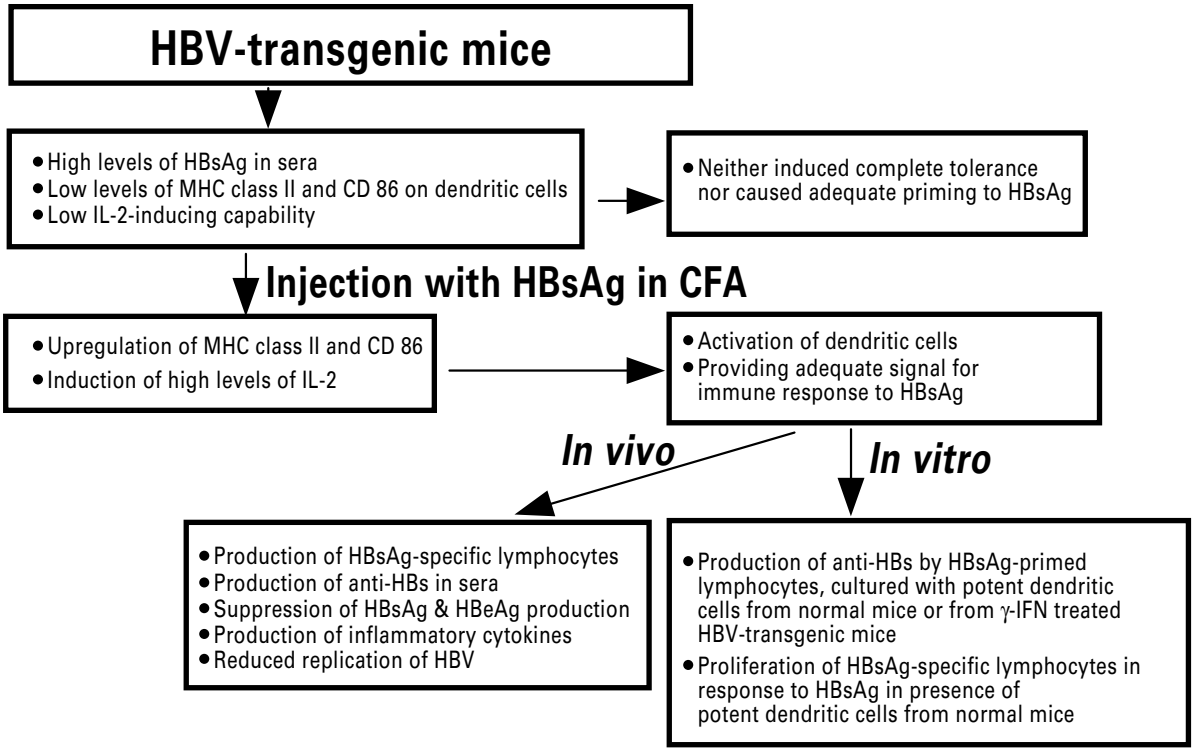

Figure 1. Activation of dendritic cells-Restoration of immune response hypothesis. Circulating HBsAg neither prime HBV-transgenic mice nor make them completely tolerant to HBsAg. Injection of HBsAg in CFA caused upregulation of MHC class II and CD86 antigens on dendritic cells, resulting in activation of dendritic cells and increased capacity to induce IL-2 production. This led to presentation of HBsAg in HBV-transgenic mice and HBsAg-primed lymphocytes were generated *in vivo*. These HBsAg-specific lymphocytes produced inflammatory cytokines and anti-HBs and resulted in reduced replication of HBV and decreased expression of HBV-related markers. The HBsAg-specific lymphocytes produced anti-HBs or proliferated in response to HBsAg when challenged *in vitro* with HBsAg in the presence of efficient dendritic cells from normal C57BL/6 mice or dendritic cells from HBV-transgenic mice, treated with γ-IFN.

mice caused neither adequate priming nor induced complete tolerance in HBV-transgenic mice.

Mechanism underlying the HBsAg-specific immune response in HBV-transgenic mice

Although production of anti-HBs and the generation of HBsAg-specific lymphocytes was seen in HBV-transgenic mice after injecting HBsAg in CFA, it remained to be elucidated why circulating HBsAg alone could not prime HBV-transgenic mice sufficiently. The difference between circulating HBsAg and injection of HBsAg in CFA in HBV-transgenic mice was studied further to find out the clinical significance of these observations. In order to have an adequate immune response, antigens enters the host and should be recognized by APCs. Recognition of antigen by APCs leads to processing of antigen to peptide fragments, and the APCs with peptides in association with self MHC should present the antigen to MHC-restricted lymphocytes. Recognition of antigen in association with self-MHC is essential, and proper recognition would lead to generation of the second signals, mainly delivered by costimulatory molecules, and this would lead to T cell activation and production of immune regulatory cytokines and so an immune response is initiated (Steinman 1991). We have proposed how injection of HBsAg in CFA in HBV-transgenic mice might influence the immune response of these mice to HBsAg (Figure 1). The defective APC capability of dendritic cells was found to be due to lower levels of MHC class II molecules and CD 86 antigens on dendritic cells. Both of these molecules are vital for initiation of the immune response. We found that circulating HBsAg did not cause an upregulation of these vital antigens on dendritic cells. But, injection of HBsAg in CFA caused an upregulation of MHC class II and CD 86 antigens on dendritic cells, and the expression of these antigens peaked 3 days after injecting HBsAg in CFA (Table 3). In addition to this, injection of HBsAg in CFA in HBV-transgenic mice caused production of significantly higher levels of IL-2. We speculate that the upregulation of MHC class II and CD 86 and increased production of IL-2 following injection with HBsAg in CFA is vital for the initiation of the immune response in HBV-transgenic mice. Thus, the circulating HBsAg did not initiate an immune response because it could not provide adequate signals for the activation of APCs. However, the injection of HBV-transgenic mice with HBsAg in CFA provided the vital signals by upregulating the expression of MHC class II and CD 86 antigens on dendritic cells, and initiated the immune response to HBsAg (Matziger 1994).

Understanding the immune response in HBV-transgenic mice and its application for therapeutic purpose

It became evident that the activation of APCs is vital for the initiation of an immune response in HBV-transgenic mice to HBsAg, which has so far been considered as a tolerogen. The kinetics of HBsAg-induced upregulation of MHC class II and CD 86 antigens on dendritic cells revealed that injection of HBV-transgenic mice with HBsAg in CFA caused the peak level of these antigens 3 days after injection, and enhanced expression persisted for a maximum of 21 days before it returned to almost prevaccinated levels. This led us to hypothesize that if the expression of MHC class II and CD 86 can be persistently upregulated by injecting HBsAg in CFA periodically in HBV-transgenic mice, there might be a persistent activation of dendritic cells, which may be sufficient to induce an HBsAg-specific immune response for a prolonged period. We injected HBV-transgenic mice with HBsAg in CFA once a month for 12 consecutive months, and this new approach to therapy was termed 'vaccine therapy' (Akbar *et al*. 1997). A 12-month regimen of vaccine therapy, in which HBV-transgenic mice with high levels of HBsAg, HBeAg and HBV DNA were injected i.p. once a month with vaccine containing HBsAg

Table 3. Upregulation of surface markers on spleen dendritic cells from HBV-transgenic mice due to vaccination

	Mean fluorescence intensities	
Dendritic cells	MHC class II	CD86
Normal C57BL/6 Mice (untreated)	454.0 ± 85.0	232.0 ± 54.0
Transgenic mice (untreated)	164.0 ± 65.0	132.0 ± 44.0
Transgenic mice (injected with HBsAg in CFA)	394.0 ± 145.0*	224.0 ± 56.0*

Flow cytometric analyses of MHC class II, and CD 86 antigens on spleen dendritic cells isolated from untreated normal, untreated HBV-transgenic and HBV-transgenic mice, 3 days after injecting HBsAg in CFA. Expression of MHC class II and CD 86 are shown as mean ± standard deviation of mean fluorescence intensities ($n = 5$). *Injection of HBV-transgenic mice with HBsAg in CFA resulted in significant upregulation of both MHC class II and CD 86 antigens on spleen dendritic cells.

HBsAg		HBeAg		Anti-HBs	
B	A	B	A	B	A
2^{8*}[32]$^{\#}$(+ ve)	2^{1}[25] (– ve) 2^{4}[4] (+ ve) 2^{5}[3] (+ ve)	2^{4}[32](+ ve)	2^{1}[30](– ve) 2^{3}[2](+ ve)	$< 2^{2}$[32](– ve)	2^{4}[5](+ ve) $< 2^{2}$[27](– ve)

Table 4. Antiviral and immunomodulatory potentialities of vaccine therapy in HBV transgenic mice

* Titres of HBsAg, HBeAg and anti-HBS were shown in Reverse passive haemaoagglutination and passive haemoaggutination methods, and titres $> 2^{2}$ indicated positivity. B: Before the start of vaccine therapy, A: after the end of 12 months of vaccine therapy. $^{\#}$[] indicates the numbers of HBV-transgenic mice. HBsAg and HBeAg became completely negative in 25 and 30 of 32 of vaccine recipient HBV-transgenic mice and 5 HBV-transgenic mice developed circulating anti-HBs due to vaccination.

in CFA, showed that at the end of vaccine therapy, 25 and 30 of the 32 HBV-trasngenic mice became completely negative for HBsAg and HBeAg, respectively (Table 4). Semiquantative estimation of HBV DNA by PCR showed that there was more than a 100-fold reduction of HBV DNA due to vaccination in all HBV-transgenic mice. In situ hybridization for HBV DNA also showed a decrease in signals for HBV DNA following vaccination compared with control HBV-transgenic mice receiving only CFA or receiving no special treatment (Figure 2). The most significant finding was that 5 HBV-transgenic mice developed circulating anti-HBs after vaccine therapy, whereas the control HBV-transgenic mice did not show any significant change in HBV markers. Vaccine therapy has been repeated on 4 occasions in our laboratory using HBsAg in CFA or HBsAg in

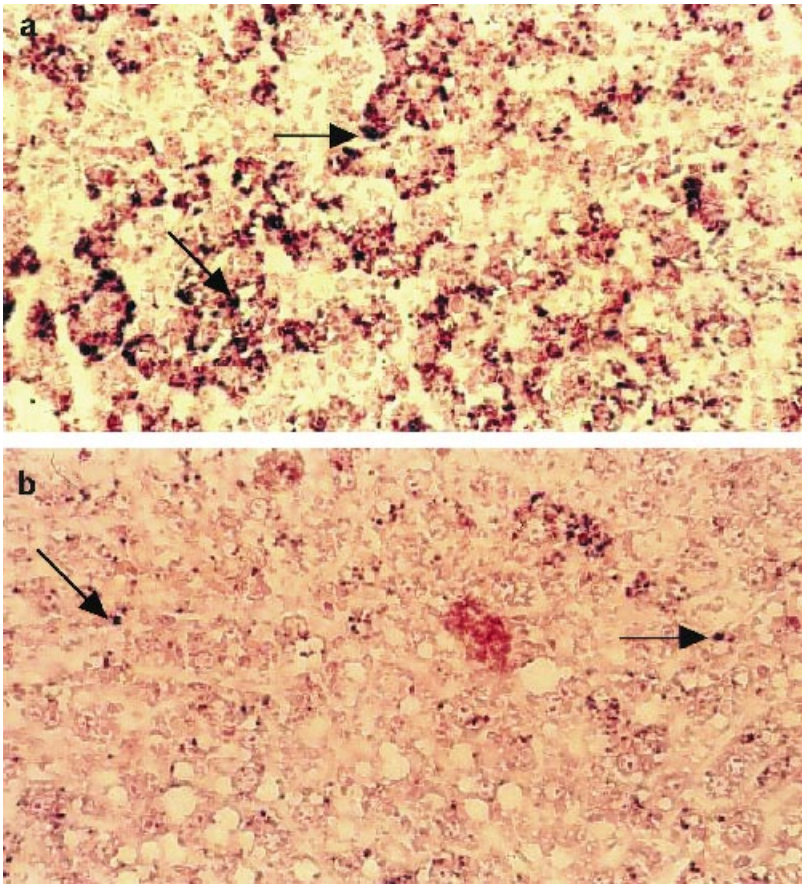

Figure 2. *In situ* hybridization for HBV DNA in the liver from an HBV-transgenic mouse (a) receiving monthly injections of CFA for 12 months (control group) or (b) receiving monthly injections of vaccine containing HBsAg in CFA for 12 months. HBV DNA was seen as black granules in the liver using a digoxigenin-labelled HBV DNA probe. A decrease in the signals for HBV DNA was seen in HBV-transgenic mice after the completion of vaccine therapy (b) compared with HBV-transgenic mice treated with only CFA (a).

aluminium gel, and we have consistently recorded the antiviral and immunomodulatory potential of vaccine therapy in 60–80% of HBV-transgenic mice.

Mechanisms underlying the therapeutic potential of vaccine therapy

Vaccine therapy was not associated with a rise in serum transaminase, and it was predicted that a noncytolytic destruction of HBV had been taking place in HBV-transgenic mice following vaccination. The noncytolytic destruction of HBV DNA has been reported in another line of HBV transgenic mice in which different inflammatory cytokines, produced by the injection of HBsAg-specific CTLs, influenced HBV replication and HBV gene expression (Chisari *et al.* 1995). We have already reported an increase of IL-2 (Akbar *et al.* 1997) after injecting HBsAg in CFA, and have recently found increased production of TNF-α and IFN-γ following vaccination in HBV-transgenic mice. It is highly likely that vaccination caused upregulation of MHC class II and CD 86 on APCs, which caused activation of APCs and induction of an immune response to HBsAg. This led to production of HBsAg-specific lymphocytes, which produced abundant amounts of IL-2, TNF-α and IFN-γ. These inflammatory cytokines contributed to noncytolytic destruction of HBV and also suppressed HBV gene expression.

Conclusion and perspective

It is now apparent that the transgenic mouse model has contributed significantly to the understanding of many aspects of HBV biology, immunology and pathogenesis. We now know that HBV is capable of replicating in mouse hepatocytes and also in other mouse cell types indicating that there is strong tissue or species-specific constraint to viral replication once the viral genome enters the cells. However, the absence of the supercoiled form of HBV DNA indicates that there might be other relevant factors to be addressed in the future.

The understanding of the pathological basis of HBsAg filament formation and ground glass hepatocytes has shown that viral gene products can be toxic to hepatocytes.

The transgenic mouse model has shown conclusively that HBV-induced liver disease has an immunological basis, and class I restricted CTLs have a critical role in this process. CTLs recognize their target on hepatocytes, and induce apoptosis. Moreover, they also cause an influx of many inflammatory cells which produce inflammatory cytokines and cause further liver damage. If this process goes unchallenged it may lead to fulminant hepatitis. The occurrence of fulminant hepatitis might be blocked by using antibodies to inflammatory cytokines.

We have also seen that CTLs are unable to recognize HBV-positive parenchymal cells outside the liver, apparently because they cannot traverse the microvascular barriers that exist at extrahepatic sites. This observation might be related to viral persistence after therapy in human HBV-carriers.

HBV-transgenic mice have also illustrated that activated CTLs and the cytokines they secrete may downregulate HBV gene expression and even control viral replication, possibly by noncytotoxic inactivation mechanisms involving the degradation of viral RNA, perhaps by degrading viral nucleocapsid and replicative intermediates without killing the cells. This basic mechanism is of vital importance because it may cause viral clearance when the immune response is rapid and it may also may cause viral persistence during chronic infection when the immune response is weak.

Experiments regarding the immune response in HBV-transgenic mice have shown that there is a very specific defect of antigen presenting function in dendritic cells and this may constitute the major defect in HBV-transgenic mice responing to HBsAg, but the contribution of factors like construction, promoter, expression product and insertion sites need further study. The costimulatory molecules and MHC molecules are lower on spleen dendritic cells from a line of HBV-transgenic mice compared with age, sex and histocompatibility-matched normal C57BL/6, the genetic background on which the transgenic mice were produced. This low expression of restriction and costimulatory molecules has been responsible for the low or inadequate response to T-cell-dependent antigens, such as KLH and HBsAg. This observation was further substantiated when the defect in the immune response could be overcome *in vitro* by using dendritic cells from normal C57/BL 6 mice that expressed normal levels of restriction and costimulatory molecules. Treatment of HBV-transgenic mice with γ-IFN restored the immune response to KLH by upregulating APC function.

The availability of transgenic mice allowed to examine the impact of neonatal tolerance to respond to self antigen. Our data have shown that antigenic challenge during the neonatal period does not render the host completely tolerant to that antigen. We emphasized the importance of proper antigen presentation in HBV-transgenic mice. Our data are in favour of a danger/harmless theory of immune response rather than a self/nonself theory. However, these data must be confirmed

independently in human HBV-carriers and in other lines of HBV-transgenics before being universally accepted.

The finding of the vital role of the activation of dendritic cells to overcome immune the response defect to self antigen like HBsAg was given a clinical significance by proposing a novel therapy for HBV-carriers, and vaccine therapy may be promising, not only to combat HBV infection but also for other chronic infections and malignancies.

The forgoing observations illustrate the power of transgenic mice to elucidate aspects of viral biology when they are used to address questions that cannot be otherwise approached by other existing methodologies. We are optimiztic that transgenic mice technology will clarify many additional aspects of chronic HBV infection.

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