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REVIEW ARTICLE

Immune selection during chronic hepadnavirus infection

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Abstract
Purpose Late-stage outcomes of chronic hepatitis B virus (HBV) infection, including fibrosis, cirrhosis, and hepatocellular carcinoma (HCC) result from persistent liver injury mediated by HBV antigen specific cytotoxic T lymphocytes (CTLs). Two other outcomes that often accompany chronic infection, the emergence of mutant viruses, including HBe-antigen negative (HBeAg (−)) HBV, and a reduction over time in the fraction of hepatocytes productively infected with HBV, may also result from persistent immune attack by antiviral CTLs. To gain insights into how these latter changes take place, we employed computer simulations of the chronically infected liver.
Methods Computational programs were used to model the emergence of both virus-free hepatocytes and mutant strains of HBV.
Results The computer modeling predicted that if cell-to-cell spread of virus is an efficient process during chronic infections, an HBV mutant that replicated significantly more efficiently than the wild type would emerge as the prevalent virus in a few years, much more rapidly than observed, while a mutant that replicated with the same or lower efficiency would fail to emerge. Thus, either cell-to-cell spread is inefficient or mutants do not replicate appreciably more efficiently than wild type. In contrast, with immune selection and a higher rate of killing of hepatocytes infected with wild-type virus, emergence of mutant virus can be explained without the need for a higher replication rate. Immune selection could also explain the emergence of virus-free hepatocytes that are unable to support HBV infection, since they should have a lower turnover rate than infected hepatocytes.

Keywords Chronic hepatitis B · Immune selection · HBeAg (−) HBV · Hepatocyte clones

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Introduction

Hepadnaviruses, of which human HBV is the prototype, replicate by reverse transcription of an RNA intermediate, the pregenome. Hepatocytes are the main cell type infected and following infection, the approximately 3-kbp relaxed circular (RC) hepadnavirus genome enters the nucleus and is converted into a covalently closed circular DNA (cccDNA) [1–4]. cccDNA serves as the template for all of the virus mRNAs, including the pregenome. cccDNA molecules, which bind histone proteins [5], are highly stable and are commonly detected at 5–50 copies per infected hepatocyte.

In transient hepadnavirus infections, >95% of hepatocytes are often infected. Since hepadnavirus infection is not cytopathic, elimination of hepadnavirus-infected hepatocytes requires immune attack by virus antigen-specific CTLs, leading to the elimination of cccDNA directly by

hepatocyte death or indirectly during compensatory hepatocyte proliferation [6]. Whether significant cccDNA loss occurs in the absence of hepatocyte turnover (i.e., hepatocyte death and compensatory proliferation) remains unclear [7]. We and others have reported that resolution of transient hepadnavirus infections involves immune-mediated attack on large numbers of hepatocytes and significant turnover of the hepatocyte population [6, 8], with turnover of up to 10% of the hepatocyte population per day [6, 9, 10].

During chronic HBV infection, the percentage of HBV-infected hepatocytes often begins with >95% of hepatocytes infected but can decrease to 10–50% or fewer. In chronic infections, hepatocyte turnover is of the order of 1–5% per day or less [11, 12]. Thus, in contrast to transient HBV infections, chronic infections appear to involve persistent immune attack on infected hepatocytes, which can cause substantial clinical disease, but is nonetheless inadequate to resolve the infection [13]. In the noncirrhotic liver, replacement of hepatocytes occurs by division of other hepatocytes, and not by liver progenitor or stem cells and it is assumed, as in the uninfected liver [14], that most if not all infected hepatocytes have an equal probability of dividing to maintain liver mass.

Clinical studies have suggested that the natural course of chronic HBV infection consists in many patients of four phases: immune tolerance, immune clearance (hepatitis B e antigen [HBeAg]-positive chronic hepatitis), inactive carrier state, and reactivation (HBeAg-negative chronic HBV) [15–17]. Particularly when acquired in childhood, chronic HBV infections involve an initial immune tolerance phase with widespread infection of hepatocytes, high levels of viremia, mild immune responses, and minimal inflammation. The immune tolerance phase may progress in some patients to an immune clearance phase in which levels of viremia can fluctuate, and inflammation and liver injury become more obvious, reflecting a more aggressive immune response to the infection. The immune clearance phase may in some cases lead to a reduction of viremia to virtually undetectable levels [18]. Liver disease may become quiescent, accompanied by mild chronic hepatitis and is classified as the inactive carrier phase of HBV infection. Finally, reactivation of liver disease can also occur.

During the immune clearance phase in particular, there may be a switch in the predominant form of HBV in the liver, from wild type to HBeAg (−) mutants. These mutants are unable to make HBeAg, a virus secretory protein that is believed to suppress antiviral CTL responses to the HBV core protein (HBcAg), with which it shares immunodominant epitopes [19]. Although the mechanisms underlying this switch are uncertain, it is believed to reflect a higher rate of killing of hepatocytes infected by wild-type HBV as compared to hepatocytes infected by HBeAg (−) HBV, as discussed below. The conversion is often accompanied by the appearance of anti-HBe antibodies.

While the emergence of HBeAg (−) HBV has been observed in many clinical studies, another possible example of immune selection by antiviral CTLs has been less considered. Typically, a large fraction of hepatocytes in the chronically HBV infected liver, including, but not limited to foci of altered hepatocytes (FAH) [20, 21], do not contain detectable levels of virus antigens and nucleic acids [22–32], suggesting that these hepatocytes do not support HBV infection. Alternatively, some of these hepatocytes may express HBV surface antigen (HBsAg) in the absence of virus replication or expression of HBcAg. Since the strongest selective pressure in the liver of an HBV carrier is against infected hepatocytes, it seems probable that emergence of apparently virus-free hepatocytes during a chronic infection, or hepatocytes expressing HBsAg, but not supporting HBV replication, must also reflect a host immune response that selects for hepatocytes that have lost the ability to express all, or a major subset of, HBV antigens.

In the following section, we show using a simple computational model how killing of infected hepatocytes by antiviral CTLs may easily explain the emergence of virus-free hepatocytes. In a subsequent section, we use additional computational models to evaluate possible explanations for the emergence of HBeAg (−) HBV as the prevalent virus strain in chronic HBV infections. These models support the current idea that negative immune selection by antiviral CTLs may also be the major cause for emergence of HBeAg (−) HBV, although as discussed below, other mechanisms may also make significant contributions.

Results

Selection of virus-negative hepatocytes

Introduction

A large number of studies, some of which are cited above, support the idea that virus-free hepatocytes emerge during the course of a chronic HBV infection. It is possible that these hepatocytes contain integrated and cccDNA. Nevertheless, the level of expression of HBcAg and HBV DNA, both considered markers of a productive infection when found in the cytoplasm, is too low to be detected by conventional assays.

This is illustrated in Fig. 1 and Table 1. Liver tissue from a group of four patients with chronic HBV infection was collected 13–14 years following renal transplant and

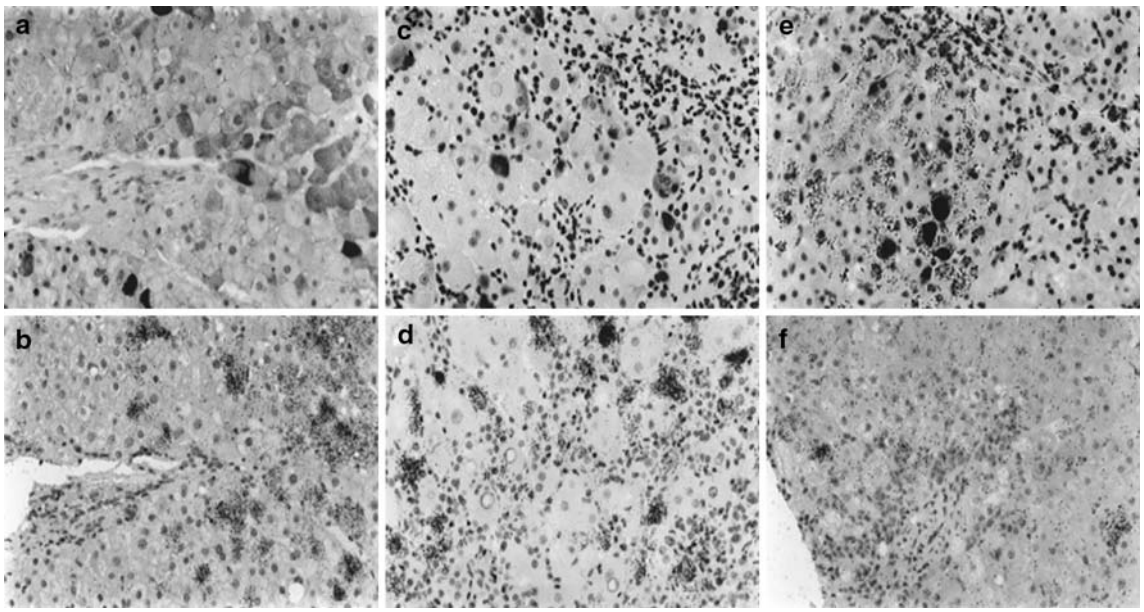

Fig. 1 Detection of HBsAg (**a**, **c**, **e**) and HBV DNA (**b**, **d**, **f**) in liver tissue from renal transplant patients with chronic HBV infection. (**a**, **b**) Patient 2, with mild chronic active hepatitis (CAH) and developing cirrhosis. Cytoplasmic and membranous HBsAg and cytoplasmic HBV DNA were detected in hepatocytes that were each distributed randomly throughout the lobule; groups of hepatocytes with increased HBsAg and HBV DNA were seen in the periportal regions of the lobule. Many virus-free hepatocytes were also observed. (**c**, **d**) Patient 3, with marked CAH and established cirrhosis. The percentage of HBsAg and HBV DNA positive hepatocytes was highest in areas of acinar regeneration and at the periphery of regenerative nodules. HBsAg and HBV DNA containing hepatocytes were seen near areas of piecemeal necrosis. (**e**, **f**) Patient 4, with mild CAH and established cirrhosis. Small foci of hepatocytes were observed containing cytoplasmic HBsAg and HBV DNA. HBsAg was detected by immuno staining of liver tissue fixed with ethanol/acetic acid [33]. Nuclei were counterstained with hematoxylin. HBV DNA was detected by in situ hybridization with an ^{125}I labeled HBV DNA probe. Autoradiographic exposure, 168 h, stained with hematoxylin and eosin. Results are summarized in Table 1. Magnification, 200×

was studied by immunostaining for the presence of membranous and cytoplasmic HBsAg, cytoplasmic and nuclear HBcAg, and by in situ hybridization for HBV DNA [33]. As can be seen in Table 1, all patients were serum HBV DNA positive (indicating ongoing HBV viremia), but many hepatocytes in different liver lobules had low or undetectable levels of HBsAg, HBcAg, and HBV DNA (Fig. 1). In these patients, HBV-free hepatocytes represented up to 40–90% of hepatocytes despite the presence of ongoing viremia. The factors that prevent HBV replication in these hepatocytes are unknown, but we hypothesize that they arise in the liver due to immune escape from the antiviral immune response.

A similar phenomenon has been observed in woodchucks chronically infected with woodchuck hepatitis virus (WHV), where clusters of apparently WHV free hepatocytes and scattered hepatocytes expressing little or no WHV core antigen are readily detected (Fig. 2). As in humans, some of the foci of virus-free hepatocytes in the woodchuck liver clearly represent FAH, often considered to be preneoplastic [34–38], whereas virus-free hepatocytes in other foci appear to have a normal morphology [36]. In contrast, virtually all hepatocytes can be infected during a typical transient WHV infection [7, 10].

Computational modeling suggests that immune escape could lead to the emergence of large numbers of virus-free hepatocytes

Persistent hepatitis with an elevated level of hepatocyte turnover due to killing of infected hepatocytes by antiviral CTLs appears to characterize chronic HBV infections. There is no totally reliable measurement of hepatocyte turnover rates in the chronically infected liver, but at least some estimates put it in the range of 1–5% per day [11, 12]. This contrasts with the levels of hepatocyte turnover in a healthy, uninfected liver, which are generally in the order of 0.01–0.1% per day (here we used 0.05%). Thus, hepatocytes in a chronically infected liver that are virus free or have lost the ability to express HBV, and therefore evade immune attack, should have a strong survival advantage compared to productively infected hepatocytes. Both virus-infected and virus-free hepatocytes contribute to cell replacement in the chronically infected liver. Since liver mass is maintained even during infection of the entire hepatocyte population, it seems likely that both virus-infected and virus-free hepatocytes respond equally to signals, to divide to maintain the hepatocyte population. The consequence would be that virus-free hepatocytes

Table 1 Analysis of liver biopsy samples from renal transplant patients

Patient details	No.		1	2	3	4
	Age/sex		29F	62M	62M	52F
Serum markers	HBsAg[a]		+	+	+	+
	Anti-HBs[a]		−	−	−	−
	HBeAg[a]		+	+	−	−
	Anti-HBe[a]		+	NT[f]	NT	NT
	HBV DNA[b]		+	+	+	+
Liver markers	HBsAg[c]	M	80–100	100	90	50–100
		C	80–100	100	100	0–2–10
	HBcAg[d]	N	30–60	10	13–20	0
		C	5–50	10–50	0–10	1
	HBV DNA[d]	% +ve	50–100	50–80	10–80	0–5
		Signal	2+ to 3+	1+ to 4+	1+ to 4+	1+ to 2+
Liver histology[e]	GGC		3+	2+	1+	1+
	PMN		−	1+	3+	1+
	CIRR		−	D	E	E
	Diagnosis		CLH	CAH	CAH	CAH

[a] HBV serum antigen and antibody markers detected by immunoassays

[b] Serum HBV DNA detected by dot-blot hybridization

[c,d] HBsAg detected in hepatocyte membranes (M) and cytoplasm (C), and HBcAg detected in hepatocyte nuclei (N) and cytoplasm (C) by IF and immunoperoxidase staining in sections of both frozen and EAA-fixed liver tissue, and expressed as % positive hepatocytes in different fields

[d] HBV DNA detected by in situ hybridization in the cytoplasm of hepatocytes and expressed as % positive (+ve) hepatocytes per field; quantitated visually and expressed on a scale of 1+, 2+, 3+, and 4+. HBV DNA was detected in sections of both frozen and ethanol/acetic acid-fixed liver tissue

[e] Liver histology: ground-glass hepatocytes (GGC) and piecemal necrosis (PMN), both judged visually and rated on a scale 1+, 2+, 3+, and 4+; CIRR = cirrhosis, D = Developing cirrhosis, E = Established cirrhosis. Diagnosis: CLH = chronic lobular hepatitis, CAH = chronic active hepatitis

[f] NT = not tested

undergo clonal expansion, not because they have a higher growth rate than infected hepatocytes, but because they have a lower death rate.

To examine the consequences of a lower death rate, we have computed clonal expansion of virus-free hepatocytes in a chronically infected liver. Infected hepatocytes were assumed to be killed at rates of 0.5, 1.0, 2.5, or 5.0% per day, while uninfected hepatocytes are killed at a rate of 0.05% per day. All hepatocytes were assumed to have an equal probability of dividing to maintain liver cell mass. In Fig. 3, we have plotted the change in the number of virus-free hepatocytes, which were arbitrarily assumed to be present at an initial frequency of 10^{-5}. Even when infected hepatocytes were killed at the relatively low rate of 0.5% per day (kd = 0.005), uninfected, virus-free hepatocytes which were killed at a rate of 0.05% per day (kd = 0.0005) still had a 10-fold survival advantage that allowed them to repopulate the entire liver in 8–9 years. Obviously, since the HBV carrier state persists, this does not happen. Some possible reasons are that such hepatocytes are not initially as frequent as supposed in Fig. 3 and, in addition that the structure of the liver lobules imposes constraints on clonal expansion. An additional constraint may arise if hepatocytes that divide in response to CTL killing are preferentially located immediately adjacent to the dying hepatocyte. This would impose a limit on the rate of clonal expansion because division within a virus-free focus would then be largely restricted to hepatocytes near the border with infected hepatocytes. Division in the interior of the focus would be lower because hepatocyte death was also lower. It can be estimated that only 20% of a spherical clone of 10,000 virus-free hepatocytes would be located at the surface and potentially adjacent to infected hepatocytes.

While virus-free FAH and foci of virus-free, but morphologically normal hepatocytes [20, 21, 36–38] would appear to fulfill the role described in the model (Fig. 3), it is not yet known if these histologic entities are clonal. It is possible to look for clonal expansion of hepatocytes in the chronically infected liver by assaying for virus DNA integrated at random sites in the host genome. A double-stranded linear DNA formed as a by-product during hepadnavirus replication appears to be the preferential precursor to integrated virus DNA, with insertion occurring

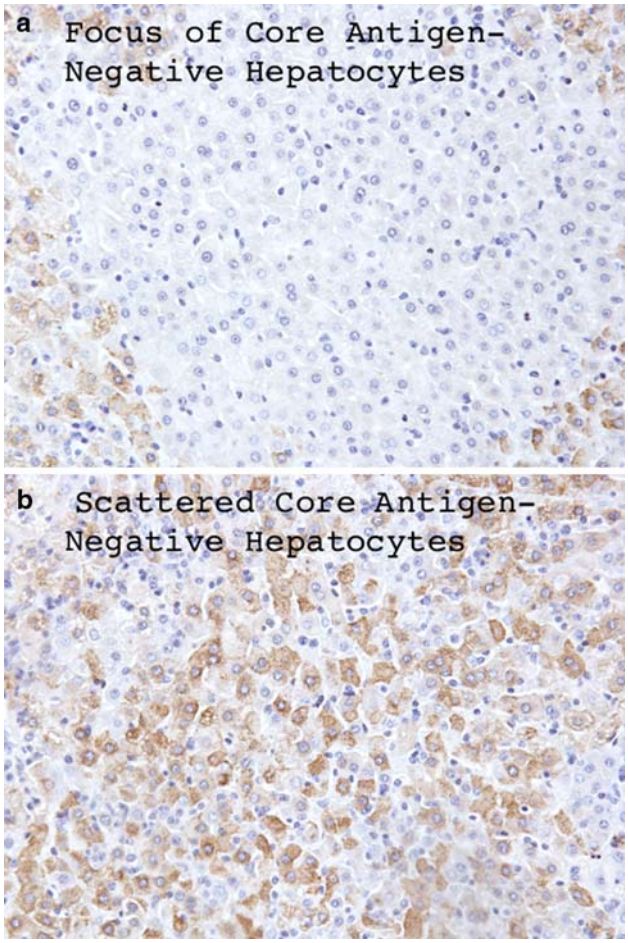

Fig. 2 Expression of WHV core antigen in the chronically infected liver. A liver tissue section from a 27-month-old chronically WHV-infected woodchuck was subjected to immuno staining for detection of WHV core antigen, and counterstained with hematoxylin [10]. (**a**) Shows a focus of hepatocytes in which WHV core antigen was undetectable. An adjacent area of the liver in which the majority of hepatocytes contain detectable levels of WHV core antigen is shown in (**b**). Magnification, 200×

by nonhomologous recombination [39–45]. It was found that clones of >1,000 hepatocytes comprised at least 1% of the liver of 27-month-old WHV carrier woodchucks, a degree of clonal expansion much too high to be explained by clonal growth due to random hepatocyte turnover [46]. Thus, a selection process may be inferred to be responsible for the observed clonal expansion [46]. Only about 1% of the hepatocytes within these livers contained integrated WHV DNA that was detectable by the assays used. Thus, it is likely that the fraction of the liver comprised of these large clones of hepatocytes is much greater than 1%. It should be possible to determine, using integrated WHV and HBV DNA, if the virus-free hepatocyte foci that have been defined histologically correspond to such clones of hepatocytes.

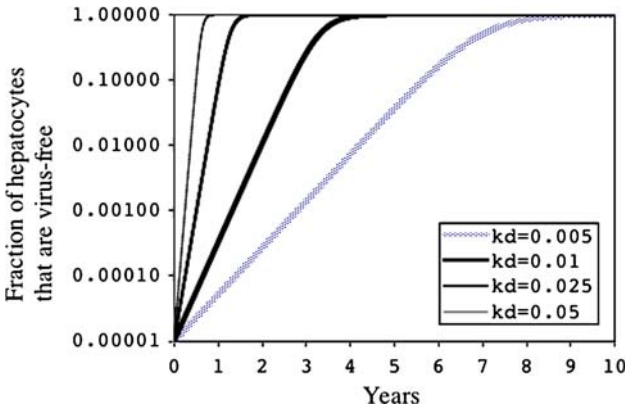

Fig. 3 Immune selection of virus-free hepatocytes in the chronically infected liver. In the model "liverfix" a small fraction of hepatocytes (i.e., uninfected) are killed by CTLs at a lower rate than the majority of hepatocytes. Let $H(0)$ be the initial liver fraction composed of such privileged liver hepatocytes. $p1$ is the fraction of ordinary infected hepatocytes that are killed per day and $p2$, less than $p1$, is the fraction of privileged hepatocytes killed per day. We assume that both hepatocyte types undergo mitosis with equal probability and that hepatocytes proliferate each day to maintain liver mass. Under these conditions, the privileged hepatocyte fraction increases each day according to the recursion: $H(n+1) = (1-p2)H(n)/[(1-p1)(1-H(n)) + (1-p2)H(n)]$, where $H(n)$ stands for the fraction of privileged hepatocytes on day n. (The model is available from S. Litwin [Samuel.Litwin@fccc.edu] upon request). In the figure, uninfected hepatocytes are killed daily at a rate of 0.0005 per day, while infected hepatocytes are killed daily at the rate indicated in the figure legend. Thus, if infected hepatocytes were killed at a rate of 0.01 (kd = 0.01), or 1% per day, uninfected, virus-resistant hepatocytes, if initially present at a frequency of 10^{-5}, would become the dominant population of hepatocytes in the liver after 3–4 years

Summary

On the basis of the analysis in Fig. 3, it is easy to see that clones of virus-free hepatocytes could emerge due to immune selection directed against hepatocytes that continue to produce high levels of HBV or WHV. A related question, addressed below, is whether this same model could explain the emergence of HBeAg (−) HBV during the course of a chronic HBV infection.

Emergence of HBeAg (−) HBV

Introduction

Chronic HBV infection, at least in the immune tolerance phase, is characterized by the presence of HBeAg in the serum. Early studies suggested a strong correlation between serum HBeAg and high-titer viremia. For instance, it was observed that HBsAg-positive women who were also HBeAg-positive readily transmitted HBV to their newborn, whereas the risk of transmission was much lower if antibodies to HBeAg, rather than HBeAg, were prevalent in the serum [47]. Thus, anti-HBe antibodies appeared to be a

marker of seroconversion to a lower viremia or, in some cases, to a nonviremic state in which virus production in the liver had been largely terminated.

Conversion from HBeAg to anti-HBe antibodies was also found to be a common marker of spontaneous clearance of chronic infections, of interferon-alpha and nucleoside analog induced cures of infection, and of recovery from transient infections. However, it quickly became clear that this correlation was not always correct, some patients converted from serum HBeAg to anti-HBe antibodies without clearing their infection and others converted from HBeAg positive to HBeAg (−) without production of anti-HBe antibodies, again with continuing virus production and, especially, evidence of continuing active liver disease (see [15, 16] for review). It was originally believed, since many of these patients presented with enhanced disease activity, that loss of serum HBeAg might be causal. Studies with a mouse model suggest that HBeAg might induce tolerance and suppress CTL responses against HBcAg [19], and some evidence from the woodchuck model of neonatal transmission suggests that WHV e antigen might be necessary to establish a chronic infection [48]. Thus, loss of serum HBeAg during a chronic infection might allow a more vigorous immune response to at least one class of virus antigens, those shared between HBeAg and HBcAg.

However, our immediate concern is not the function of HBeAg in HBV infections, which remains unclear, but the more special case of how HBeAg (−) HBV can emerge from a population of mostly wild-type HBV to become the predominant virus in the liver. Conversion of wild-type to HBeAg (−) HBV can occur with as little as one nucleotide change to create a stop codon in the preCore domain, which encodes the signal peptide at the amino terminal end of the protein precursor to the secreted HBeAg [49–51]. It is this simple example of an HBeAg (−) HBV with which we will be concerned here, although we expect that the same outcomes would apply to more complex variants involving the preCore promoter. Thus, if HBeAg is essential to initiate a chronic infection, HBeAg (−) HBV mutants might be expected to be present at a frequency of approximately 10^{-4}, the spontaneous mutation rate [52], during the early, immune-tolerant phase of chronic HBV infection. Later expansion might be possible because, for instance, HBeAg is no longer needed once a chronic infection is established. Therefore, approximately four logs of enrichment would be sufficient for emergence of HBeAg (−) HBV. What mechanisms could account for this enrichment?

Forward mutation

One obvious contributor would be forward mutation. While the mutation rate constant may be approximately 10^{-4} per round of cccDNA synthesis [52], the accumulation of mutations at any particular site is also driven by the relative amounts of wild-type and mutant HBV in the liver. Since virus that has a wild-type HBeAg is initially more abundant, the fraction of HBV containing an HBeAg (−) mutation will increase as infected hepatocytes proliferate to compensate for the death of other infected hepatocytes and to maintain liver mass. If the HBeAg (−) mutation is neutral, and forward and back mutation occurs with the same rate constant, viruses containing this mutation will gradually accumulate until, given enough time, they represent 50% of the virus population. The rate at which this accumulation occurs will depend on how often cccDNA is formed, which will be influenced by a number of factors that are related to how we view chronic infections.

To explore this issue of mutant emergence, we therefore used a computational model originally developed to explore the emergence of drug-resistant strains of HBV [53]. In this computational model, the wild-type and HBeAg (−) HBV were assumed, initially, to replicate at the same rate, such that infected hepatocytes would double in number every 3 days following infection of a naïve host. cccDNA was assumed to survive mitosis, distributing to progeny hepatocytes in a binomial fashion. We considered liver turnover to be of the order of 1–5% of hepatocytes per day [11, 12]. Figure 4a shows that if infected hepatocytes were killed at a rate of 1% per day, after 30 years, the frequency of HBeAg (−) HBV mutants would only rise about 100-fold, and hepatocytes containing only HBeAg (−) HBV would represent only about 1% of the total hepatocyte population. With hepatocyte turnover of 5% per day, the percentage of hepatocytes containing only HBeAg (−) HBV would rise to 5% of the total hepatocyte population (Fig. 4d). (It should be noted that in both simulations, as a result of random distribution of cccDNA to daughter cells, HBeAg (−) HBV cccDNA is gradually segregated over multiple rounds of hepatocyte division into hepatocytes that contain no wild-type HBV.) This is also the case for the model discussed in Fig. 5. This segregation provides an additional advantage for emergence of mutant virus if immune selection, as discussed later, is driven by antiviral CTLs that selectively kill hepatocytes containing wild type HBV.

On the other hand, if HBeAg (−) HBV mutants had a replication rate twice that of wild-type HBV (i.e., the mutation is not neutral) and infected hepatocytes were killed at a rate of 1% per day, after 30 years, hepatocytes containing only mutant HBV would represent about 10% of the total hepatocyte population (Fig. 4b). With killing at 5% per day, after 30 years hepatocytes containing only HBeAg (−) HBV would represent about 40% of the hepatocyte population (Fig. 4e). Flares of acute hepatitis or a contribution from immune selection would then allow

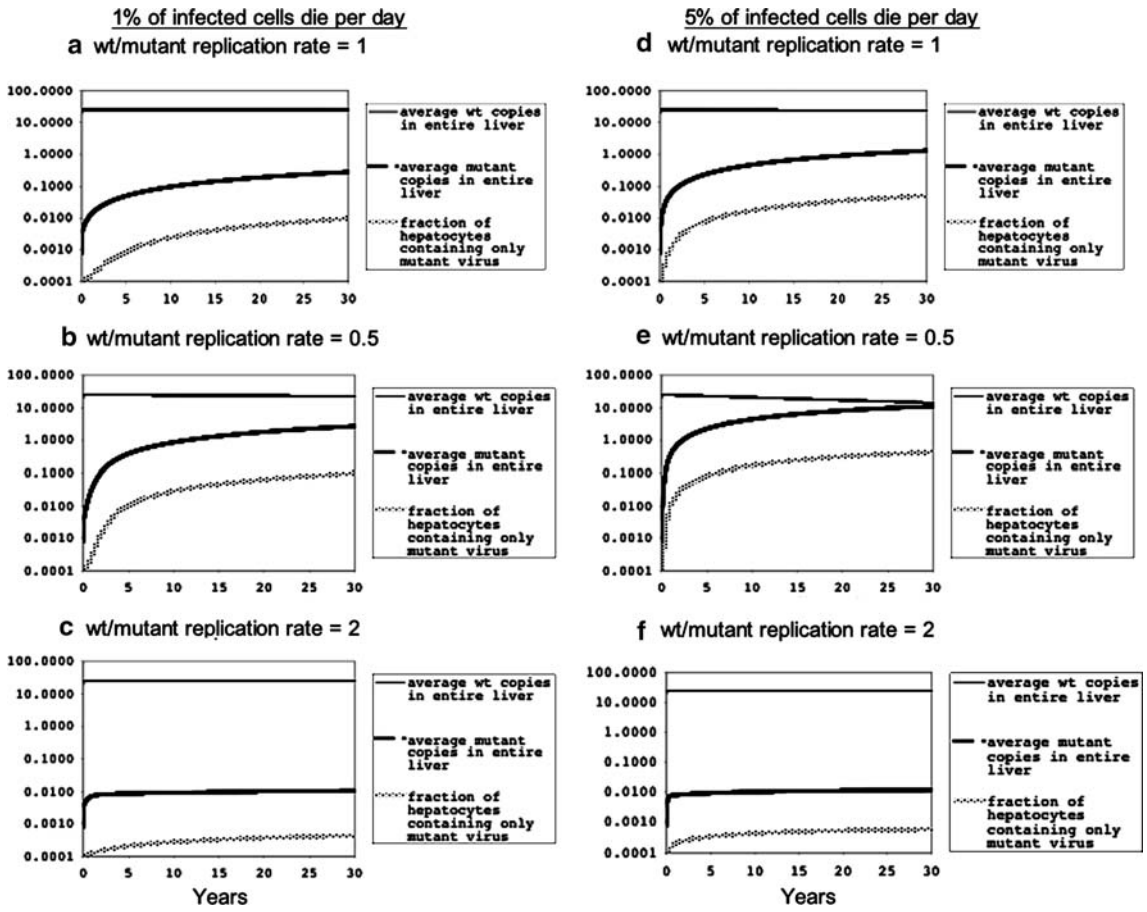

Fig. 4 Emergence of HBeAg (−) HBV due to spontaneous mutation. To compute the kinetics of emergence of HBeAg (−) HBV, the model simulates a liver of 3×10^{10} hepatocytes. (This liver size constraint does not effect the calculations reported here, since all calculations are based on fractions of a liver.) Infection status, cccDNA copy number, virus genotype (wild type, HBeAg (−) HBV), and virus production was followed in each hepatocyte as a function of the number of rounds of hepatocyte death and proliferation. Forward- and back-mutation were assumed to occur with a rate constant of 10^{-4} per nucleotide [52]. In addition, it was assumed that the hepatocyte population was maintained by proliferation of other hepatocytes, and that all hepatocytes had an equal chance of dividing. The model is described in detail in [53] and is available upon request (Samuel.Litwin@fccc.edu). (**a**) cccDNA was assumed to have a uniform distribution from 1 to 50 copies per hepatocyte, and DNA synthesis was assumed to build up cccDNA copy number exponentially so that a hepatocyte with 25 copies would reach this value after 3 days. Virus spread was assumed to be sufficient to double the number of infected hepatocytes every 3 days. Mutant virus, initially present at a frequency of 10^{-4}, was assumed to have the same replication rate as wild-type HBV. Infected hepatocytes were assumed to be killed at a rate of 1% per day and uninfected hepatocytes (e.g., resulting from cccDNA dilution when hepatocytes with a low cccDNA copy number divide) at a rate of 0.05% per day. The fraction of hepatocytes containing only mutant HBV is plotted along with the average copy number per total hepatocytes of mutant and wild-type cccDNA. (**b**) The simulation was carried out as in (**a**), except that the mutant virus was assumed to replicate twice as efficiently as the wild type. (**c**) As in (**a**), except that the mutant was assumed to replicate half as efficiently as the wild type. (**d**, **e**, **f**) As in (**a**, **b**, and **c**), respectively, except that infected hepatocytes were assumed to be killed at a rate of 5% rather than 1% per day

them to easily become dominant (see below). Finally, if the mutant replicated half as fast as wild type, hepatocytes containing solely HBeAg (−) HBV would not become a significant fraction of the total hepatocyte population (Fig. 4c, f).

We also considered the possibility that cccDNA, and its precursors, are lost when hepatocytes pass through mitosis. If as in Fig. 4a, infected hepatocytes are killed at a rate of 1% per day and both wild-type and mutant HBV replicate at the same rate, the need to reinfect postmitotic hepatocytes provides a modest increase in hepatocytes infected only with HBeAg (−) HBV, from 1% to 6% after 30 years (Fig. 5a). If the turnover of infected hepatocytes is 5% per day, hepatocytes infected only with HBeAg (−) HBV represent 20% of the hepatocyte population after 30 years (Fig. 5c). Thus, the effects of cccDNA loss during mitosis are relatively modest when the mutant and wild type replicate at the same rate. (In the simulation, we assume that hepatocytes that lose cccDNA during mitosis are reinfected by only a single virus, and that reinfection with either wild type or mutant occurs in proportion to the prevalence of each strain in the liver.) In contrast, if the mutant replicated

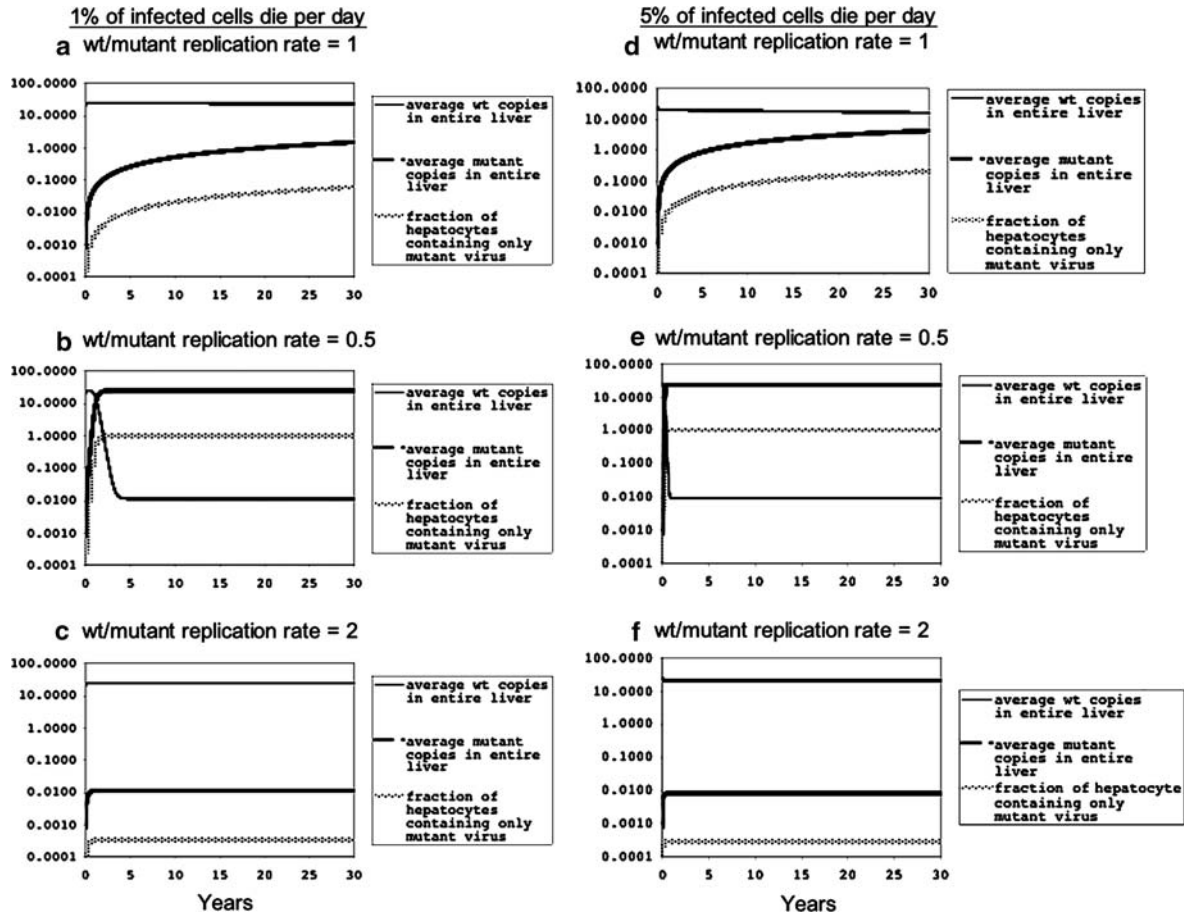

Fig. 5 Emergence of HBeAg (−) HBV due to spontaneous mutation: effect of loss of virus infections as hepatocytes pass through mitosis. Simulations in (**a**–**f**) were carried out as in (**a**–**f**), respectively, of Fig. 4, except that cccDNA was assumed to be lost and hepatocytes cured as they passed through mitosis

twice as fast as wild type HBV, it would quickly become the dominant virus in the liver (Fig. 5b, e). This would also be true if the mutant had only a slight replication advantage. For instance, with killing of 1% of infected hepatocytes per day, a virus that replicated 20% more efficiently than the wild type would emerge after approximately 5 years, and a virus with only a 5% replication advantage would emerge after approximately 15 years (not shown). In contrast, if it replicated less efficiently, it would never emerge (Fig. 5c, f).

At present it is not certain how well HBeAg (−) HBV replicates in vivo as compared to the wild-type virus from which it is derived. Yeh et al. [54] recently reported on the emergence of HBeAg (−) HBV following discontinuation of a short course of Lamivudine therapy, administered to three HBV carriers with elevated serum ALT levels. Prior to or near the start of therapy, the mutant-to-wild type ratio was about 25%. When therapy was stopped, the mutant increased to become nearly 100% of circulating virus within a few months. Following a flare of acute hepatitis, the ratio then dropped in patients as wild-type HBV again became predominant. The authors concluded that HBeAg (−) HBV replicates more efficiently than wild type. However, the data do not rule out immune selection against hepatocytes infected with wild-type HBV as a basis for the transient emergence of HBeAg (−) HBV as the predominant virus (see below). Moreover, it is difficult, without other data, to explain the reemergence of wild type if, in fact, the mutant virus replicates more efficiently. Thus, all that at present seems clear is, that loss of cccDNA during mitosis would lead to a rapid emergence of any mutant that replicated more efficiently than wild type, but this rapid emergence does not occur. Thus, it seems that either cccDNA is not lost at mitosis or the mutant does not replicate any more efficiently than the wild-type virus. Again, it is not known for certain if cccDNA survives mitosis, though some published evidence suggests this is the case [12, 55].

A high rate of hepatocyte death of 5% per day could also make a contribution to the emergence of mutant HBV (Fig. 5c). Whether this high rate is ever sustained in patients over long periods, basically through the course of their chronic HBV infection, is unknown.

In summary, forward mutation during the course of a chronic infection does not appear by itself to explain emergence of HBeAg (−) mutant viruses (Fig. 4a, d).

Forward mutation and superinfection

One way for a mutant to spread through the liver might be by superinfection of other hepatocytes. Superinfection of hepatocytes was demonstrated in one study with duck hepatitis B virus (DHBV) [56] using a DHBV-based vector that expressed green fluorescent protein. The occurrence of HBV isolates that appear to be intergenotype recombinants may also be indirect evidence of superinfection, but an equally plausible explanation is that the putative recombinants arose following simultaneous coinfection of uninfected hepatocytes rather than by superinfection. In fact, with the exception cited above, superinfection of hepatocytes has been difficult to document. The process is likely to be inefficient in as much as excessive accumulation of cccDNA does not seem to occur, most copy number estimates of cccDNA being in the range of 5–50 per hepatocyte. A study of single nuclei from DHBV-infected ducks also suggested that cccDNA copy numbers remain low [57], unlike what might be anticipated if the hepatocytes were being constantly superinfected. Hepatitis delta virus (HDV) has the HBV envelope, and its ability to superinfect the chronically HBV-infected liver might be taken as evidence that superinfection is an efficient process. However, although HDV also replicates in the nucleus, its genome is encased in HDV antigen, and not HBcAg, and it is possible that resistance to HBV superinfection occurs at a later stage than virus attachment, for example during nuclear entry of RC DNA to form new cccDNA molecules.

To investigate how superinfection might contribute to emergence of HBeAg (−) HBV, we examined the consequences of superinfection, using the computational model described in Fig. 4 [53]. In these computations, hepatocytes that accumulate 60 or more copies of cccDNA were arbitrarily assumed to die. The effect of cccDNA copy number on hepatocyte viability is not well characterized, but it is clear that unregulated accumulation of hundreds of copies of cccDNA can lead to cell death [58–60]. Since the measured average copy number of cccDNA in infected hepatocytes is generally much lower, in the range of 5–50, it is apparent that if superinfection takes place, it is not efficient enough to have a major effect on the cccDNA copy number.

Figure 6a shows the results of a computation in which superinfection was arbitrarily set to occur at a frequency of 5% per day, CTL killing of infected hepatocytes at a rate of 1% per day, and the mutant and wild-type viruses were assumed, as in Fig. 4a, to replicate at the same rate. Again as in Fig. 4, the mutation rate was set to 10^{-4}. Three conclusions are readily apparent. First, at this rate of superinfection an accumulation of cccDNA in excess of the mean of 25 copies per cell does not occur. This can be attributed to the fact that cccDNA is removed by cell death at approximately the same rate as it is added by superinfection. Second, as might have been expected, the net accumulation of mutants is not significantly altered from that which in the model can be attributed to forward mutation. And third, as a result of superinfection there is very little accumulation of hepatocytes infected only with HBeAg (−) HBV (cf. Fig. 4a); that is, superinfection overcomes the segregation of mutant and wild type virus into separate hepatocyte lineages. Thus, on the basis of this analysis it is difficult to see how superinfection could contribute to the emergence of a mutant virus that has the same replication rate, or a lower replication rate, than the wild type.

If the mutant is assumed to replicate at twice the rate of wild type, a very different picture emerges. In this case, the mutant will eventually take over in any hepatocyte lineage it infects, as new cccDNA is added to increase copy number following each round of mitosis. Thus, with the parameters used in Fig. 4b, but with a daily rate of superinfection of 5%, an HBeAg (−) mutant would become the major virus in the liver after approximately 6 years (Fig. 6b). This would occur even more rapidly if the daily rate of turnover of infected hepatocytes was 5% rather than 1% as shown in Fig. 6b, or if superinfection was more efficient.

When the simulation in Fig. 6b was carried out assuming the mutant replicated 20% better than the wild type, emergence occurred after 15 years (not shown). In contrast, a mutant virus that replicated only 5% better than the wild type did not emerge even after 30 years. Thus, it is possible to imagine a scenario in which HBeAg (−) HBV emerged in middle age because the mutant had only a minor growth advantage, perhaps 10% over wild type (not shown).

The major problem with this model is that little is known about superinfection efficiency, or about in vivo replication rates of HBeAg (−) HBV. However, with what would seem to be a low superinfection efficiency and a modest replication advantage, the mutant would quickly take over, a phenomenon not observed in patients. Thus, in our view, a model based on emergence of HBeAg (−) HBV through cell-to-cell spread via superinfection, as in Fig. 6b, is not compelling.

Flares of acute hepatitis

Flares of acute hepatitis resulting from CTL killing of infected hepatocytes sometimes occur in HBV carriers,

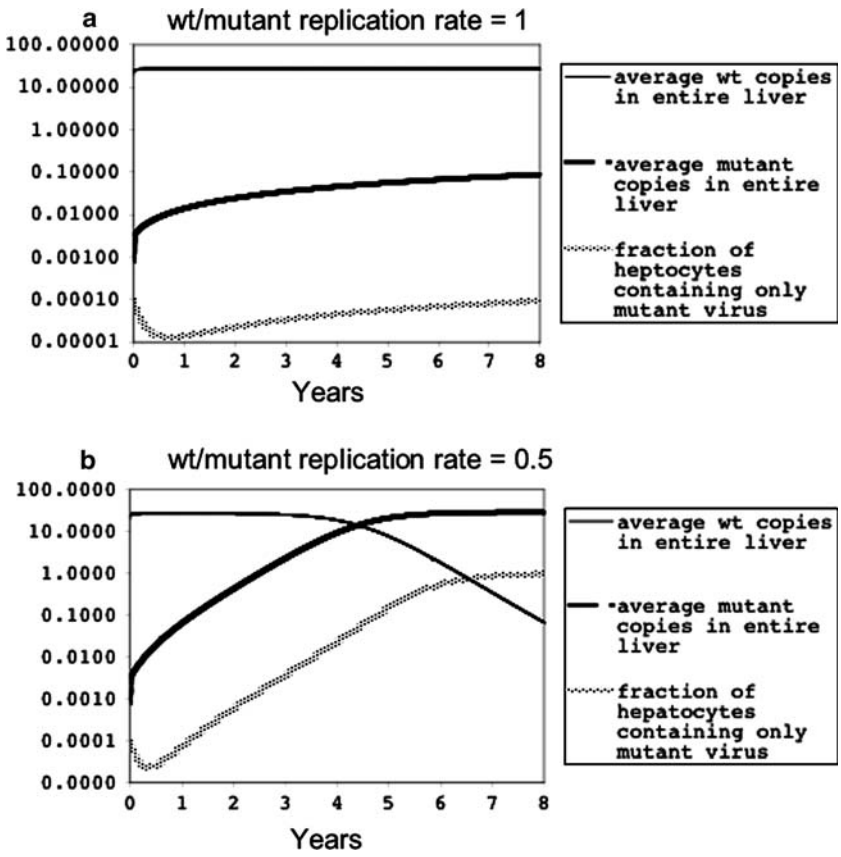

Fig. 6 Effect of spontaneous mutation and superinfection on the emergence of HBeAg (−) HBV. The simulations in (**a** and **b**) were carried out as in (**a** and **b**), respectively, of Fig. 4, except that hepatocytes were allowed to be superinfected at a rate of 5% per day

defining the so-called immune clearance phase of chronic HBV infection [15, 16]. The name may be appropriate in view of the evidence of liver damage, reflected by increases in liver enzymes in the serum and acute inflammation in the liver, but the extent to which these responses routinely lead to loss of cccDNA and elimination of infection is unknown. If we assume for the sake of discussion that HBV is eliminated from 99% of infected hepatocytes during flares of acute hepatitis, we can calculate the enrichment that would occur as wild type and mutant spread to occupy the new replication space. If the wild type and mutant replicate at the same rate, no enrichment would occur. However, if the replication rate of the mutant was 200% that of the wild type, a 2 to approximately 10-fold enrichment of the mutant would occur, depending upon whether the residual infected hepatocytes produced enough virus to immediately reinfect the virus-free hepatocytes, or if exponential expansion occurred into the virus-free population. Thus, flares of acute hepatitis occurring after, for instance, enrichment by spontaneous mutation, as illustrated in Fig. 4b and d, could lead to a situation in which the mutant became equally or more abundant than wild type. Unfortunately, as noted above, there is no definitive evidence that HBeAg (−) HBV replicates in vivo at a higher rate than the wild type, nor is it clear that flares of acute hepatitis are routinely associated with loss of cccDNA and curing of hepatocytes. Thus, acute flares do not appear so far to provide an explanation for the emergence of HBeAg (−) HBV unless there is a differential killing of wild-type infected hepatocytes during the flare (see immune selection, below).

Immune selection

The final mechanism for emergence of HBeAg (−) HBV we will consider is immune selection; that is, preferential killing by immune attack on hepatocytes expressing wild-type HBV. The consequences of small differences in death rates on hepatocyte populations are plotted in Fig. 7. Here we assume that hepatocytes that are infected, but do not express HBeAg and, presumably do not contain any wild-type cccDNA, are killed at a slightly lower rate than hepatocytes with at least one copy of wild-type cccDNA. Hepatocytes expressing HBeAg are assumed to be killed at a rate of 1% per day. It is assumed that cccDNA survives hepatocyte mitosis and is inherited by progeny hepatocytes, and that infected hepatocytes that do not make HBeAg are present initially at a frequency of 10^{-4}. With a death rate of 0.9% per day (90% of the wild type), HBeAg (−) HBV infected hepatocytes would become the major population in the liver after approximately 25 years. With a death rate

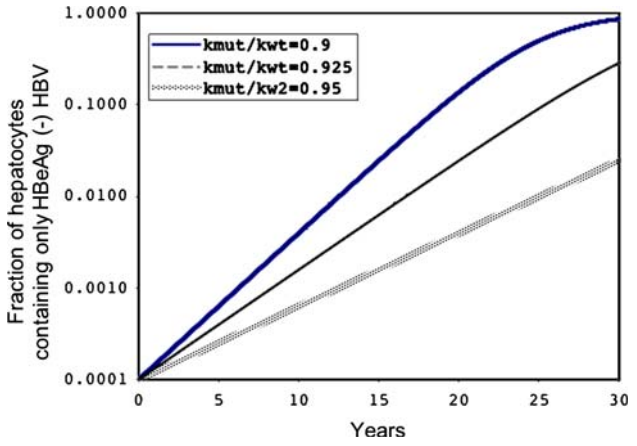

Fig. 7 Effect of small differences in hepatocyte killing by antiviral CTLs on emergence of HBeAg (−) HBV. The program liverfix, described in the legend to Fig. 3, was used to calculate the effects of small differences in death rates on emergence of clones of hepatocytes infected with HBeAg (−) HBV (without coinfecting wild-type HBV). Hepatocytes infected with wild type were assumed to be killed at a rate of 1% per day ($k_{wt} = 0.01$), and hepatocytes infected with the mutant at a slightly lower rates, k_{mut}, as indicated in the figure legend

that was 0.925% per day (92.5% of wild type), these hepatocytes would rise to approximately 30% of the infected cell population after 30 years.

Assuming the wild-type death rate was 5% per day rather than 1%, the emergence would be much faster, and even with a death rate that was as high as 0.95% (95% of the wild type), HBeAg (−) HBV hepatocytes would represent 50% of the infected hepatocyte population after 10 years (not shown).

Thus, with the proviso that hepatocyte turnover is of the order of 1–5% per day, of the four mechanisms considered here, immune selection would appear to provide the simplest explanation for the ultimate emergence of HBeAg (−) HBV. The other processes such as forward mutation and flares of acute hepatitis could also contribute to the emergence of HBeAg (−) HBV in individual patients, but immune selection would appear to be an essential step.

This rate of turnover of infected hepatocytes (1–5% per day) might be a conservative estimate for many hepatitis patients, particularly during the immune clearance phase [11], but no studies have been carried out to assess hepatocyte turnover over extended periods. Another uncertainty lies in the assumption that hepatocytes infected by HBeAg (−) HBV will be less efficiently targeted by antiviral CTLs than hepatocytes infected by wild-type HBV. Although this has not been tested with either HBV or WHV, evidence in support of this possibility was found from a study of mixed infections with wild-type DHBV and a preCore (eAg) negative mutant of DHBV [61]. Thus, the concept of immune selection and clonal expansion at least seems plausible according to the above considerations.

Summary

Even a slightly higher rate of killing of hepatocytes expressing wild type HBV as opposed to hepatocytes expressing only HBeAg (−) HBV provides a simple explanation for the emergence of the mutant as the predominant virus in the liver. The time until emergence is also determined by the absolute rates of hepatocyte killing. Thus, even with the same differential death rate, the mutant may never emerge in some patients because the total rate of hepatocyte turnover is too low, whereas in others, it may fail to emerge because CTLs do not distinguish between hepatocytes infected with wild-type and mutant HBV. In contrast to an immune selection model, models based on the forward mutation rate, acute flares, superinfection, and a higher replication rate of the mutant do not appear to provide a self-consistent explanation of the available data.

Discussion

The purpose of this modeling exercise was to find out if our current understanding of chronic hepadnavirus infections would provide an explanation for emergence of virus-free hepatocytes and of HBeAg (−) HBV. Interestingly, both outcomes were predicted by the computational models as the expected consequence of immune selection against virus-infected hepatocytes. Our expectation at the start was that immune selection would explain emergence of virus-free hepatocytes, but that it would not be able to explain emergence of HBeAg (−) HBV. This turned out to be incorrect. Given a slight survival advantage, the ultimate emergence of HBeAg (−) HBV could in large part be due to immune selection and clonal expansion of hepatocytes infected by this mutant.

While these explanations are consistent with the data, it is clear that we do not know enough about chronic infections to eliminate alternative explanations. For instance, does HBeAg (−) HBV replicate at a lower efficiency than wild-type HBV, or vice versa [54]? Does cccDNA survive mitosis or do hepatocytes have to be reinfected after passing through mitosis? How efficient is superinfection? Do flares of acute hepatitis play a critical role in the emergence of mutants? If so, how? Does differentiation of uninfected progenitor cells make a more significant contribution to hepatocyte replacement than currently appreciated, a possibility that, as in the model in Fig. 4b, could make a significant contribution to emergence of mutant viruses? At present, the best we can say is that if the mutant replicated more efficiently than wild type and if cccDNA were either lost at mitosis or if superinfection were an efficient process, the mutant would quickly become dominant over wild type (Figs. 5, 6). Since this is not the case, at least one of these

assumptions and perhaps more must be incorrect. It is likely that these issues will ultimately be clarified as more is learned about immune killing of infected hepatocytes, in vivo rates of virus replication, cccDNA survival during mitosis and in response to antiviral cytokines, and the efficiency of superinfection. Attempts to address some of these issues have already been made in animal models of HBV infection. For instance, Zhang and Summers [62] found that competition between DHBV strains with different replication rates essentially stops once the liver is fully infected, suggesting that superinfection is probably inefficient, a conclusion that could now be further tested through analysis of the cccDNA content of individual nuclei [57].

The models also have features that may have important implications for how liver disease progresses to hepatocellular carcinoma (HCC). In both examples, CTL killing would give rise to selective clonal expansion of a subset of hepatocytes. Thus, hepatocytes that were infected with HBeAg (−) HBV, or had lost the ability to support HBV infection, could undergo immune selection and extensive clonal expansion. If these hepatocytes also had pre-neoplastic mutations, they might ultimately give rise to liver tumors. In contrast, hepatocytes infected with wild-type HBV would not undergo as rapid a clonal expansion; however, as a result of random killing, some survivors would expand clonally, whereas other lineages would be lost. Interestingly, most tumors and FAH appear unable to support virus replication, suggesting that it is the immune selection for virus-free hepatocytes that is usually associated with the progression to HCC. This would make some sense since pre-neoplastic lesions that did not support virus replication would, by evading antiviral CTL, have a considerable evolutionary advantage.

Whether the clonal expansion predicted by the models occurs is currently unknown. Evidence for an extensive clonal expansion of hepatocytes has been obtained in woodchucks chronically infected with WHV [46], but this study, which relied on integrated virus DNA as a marker of hepatocyte lineages, did not correlate the clones with any histologic marker. Thus, the cause of this clonal expansion is unknown. Could virus infection per se be responsible? It is difficult to see how, in a massively infected liver, selective expansion of a subset of hepatocytes could be attributed to the activity of a normal virus protein, which would be present in all infected hepatocytes, not just a subpopulation. Thus, immune selection as the mechanism of clonal expansion of hepatocytes rather than an enhanced rate of hepatocyte proliferation induced by virus proteins at present remains the most attractive hypothesis.

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References

1. Kann M, Schmitz A, Rabe B. Intracellular transport of hepatitis B virus. World J Gastroenterol 2007;13:39–47.
2. Mason WS, Halpern MS, England JM, Seal G, Egan J, Coates L, et al. Experimental transmission of duck hepatitis B virus. Virology 1983;131:375–84.
3. Kann M. Nucleoprotein transport of HBV capsid particles. Methods Mol Med 2004;95:213–26.
4. Tagawa M, Omata M, Okuda K. Appearance of viral RNA transcripts in the early stage of duck hepatitis B virus infection. Virology 1986;152:477–82.
5. Newbold JE, Xin H, Tencza M, Sherman G, Dean J, Bowden S, et al. The covalently closed duplex form of the hepadnavirus genome exists in situ as a heterogeneous population of viral minichromosomes. J Virol 1995;69:3350–7.
6. Summers J, Jilbert AR, Yang W, Aldrich CE, Saputelli J, Litwin S, et al. Hepatocyte turnover during resolution of a transient hepadnaviral infection. Proc Natl Acad Sci USA 2003;100:11652–9.
7. Wieland SF, Spangenberg HC, Thimme R, Purcell RH, Chisari FV. Expansion and contraction of the hepatitis B virus transcriptional template in infected chimpanzees. Proc Natl Acad Sci USA 2004;101:2129–34.
8. Murray JM, Wieland SF, Purcell RH, Chisari FV. Dynamics of hepatitis B virus clearance in chimpanzees. Proc Natl Acad Sci USA 2005;102:17780–5.
9. Jilbert AR, Wu T-T, England JM, de la Hall PM, Carp NZ, O'Connell AP, et al. Rapid resolution of duck hepatitis B virus infections occurs after massive hepatocellular involvement. J Virol 1992;66:1377–88.
10. Kajino K, Jilbert AR, Saputelli J, Aldrich CE, Cullen J, Mason WS. Woodchuck hepatitis virus infections: very rapid recovery after a prolonged viremia and infection of virtually every hepatocyte. J Virol 1994;68:5792–803.
11. Nowak MA, Bonhoeffer S, Hill AM, Boehme R, Thomas HC, McDade H. Viral dynamics in hepatitis B virus infection. Proc Natl Acad Sci USA 1996;93:4398–402.
12. Zhu Y, Yamamoto T, Cullen J, Saputelli J, Aldrich CE, Miller DS, et al. Kinetics of hepadnavirus loss from the liver during inhibition of viral DNA synthesis. J Virol 2001;75:311–22.
13. Rehermann B, Pasquinelli C, Mosier SM, Chisari FV. Hepatitis B virus (HBV) sequence variation of cytotoxic T lymphocyte epitopes is not common in patients with chronic HBV infection. J Clin Invest 1995;96:1527–34.
14. Kennedy S, Rettinger S, Flye MW, Ponder KP. Experiments in transgenic mice show that hepatocytes are the source for postnatal liver growth and do not stream. Hepatology 1995;22:160–8.
15. Feld JJ, Heathcote EJ. Hepatitis B e antigen-positive chronic hepatitis B. natural history and treatment. Semin Liver Dis 2006;26:116–29.
16. Hadziyannis SJ, Papatheodoridis GV. Hepatitis B e antigen-negative chronic hepatitis B: natural history and treatment. Semin Liver Dis 2006;26:130–41.

17. Seeger C, Zoulim F, Mason WS (2006) Hepadnaviruses. In: Knipe DM, Howley PM, Griffin DE, Lamb RA, Martin MA, Roizman B, Straus SE (eds) Fields virology, 5th edn. pp 2977–3029.
18. Evans AA, O'Connell AP, Pugh JC, Mason WS, Shen FM, Chen GC, et al. Geographic variation in viral load among hepatitis B carriers with differing risks of hepatocellular carcinoma. Cancer Epidemiol Biomarkers Prev 1998;7:559–65.
19. Chen MT, Billaud JN, Sallberg M, Guidotti LG, Chisari FV, Jones J, et al. A function of the hepatitis B virus precore protein is to regulate the immune response to the core antigen. Proc Natl Acad Sci USA 2004;101:14913–8.
20. Govindarajan S, Conrad A, Lim B, Valinluck B, Kim AM, Schmid P. Study of preneoplastic changes in liver cells by immunohistochemical and molecular hybridization techniques. Arch Path Lab Med 1990;114:1042–5.
21. Bannasch P, Haertel T, Su Q. Significance of hepatic preneoplasia in risk identification and early detection of neoplasia. Toxicol Pathol 2003;31:134–9.
22. Chu CM, Yeh CT, Chien RN, Sheen IS, Liaw YF. The degrees of hepatocyte nuclear but not cytoplasmic expression of hepatitis B core antigen reflect the level of viral replication in chronic hepatitis B virus infection. J Clin Microbiol 1997;35:102–5.
23. Gowans EJ, Burrell CJ, Jilbert AR, Marmion BP. Cytoplasmic (but not nuclear) hepatitis B virus (HBV) core antigen reflects HBV DNA synthesis at the level of the infected hepatocyte. Intervirology 1985;24:220–5.
24. Gowans EJ, Burrell CJ. Widespread presence of cytoplasmic HBcAg in hepatitis B infected liver detected by improved immunochemical methods. J Clin Pathol 1985;38:393–8.
25. Hirohashi S, Shimosato Y, Ino Y, Kishi K. Distribution of hepatitis B surface and core antigens in human liver cell carcinoma and surrounding nontumorous liver. J Natl Cancer Inst 1982;69:565–8.
26. Hsu H-C, Wu T-T, Wu M-Z, Wu C-Y, Chiou T-J, Sheu J-C, et al. Evolution of expression of hepatitis B surface and core antigens (HBsAg, HBcAg) in resected primary and recurrent hepatocellular carcinoma in HBsAg carriers in Taiwan. Cancer 1988;62:915–21.
27. Kojima M, Udo K, Takahashi Y, Yoshizawa H, Tsuda F, Itoh Y, et al. Correlation between the titer of antibody to hepatitis B core antigen and presence of viral antigens in the liver. Gastroenterol 1977;73:664–7.
28. Lamothe F, Laurecin-Piche J, Cote J, Guevin R, Viallet A, Richer G. Detection of surface and core antigens of hepatitis B virus in the liver of 164 human subjects. A study by immunoperoxidase and orcein staining. Gastroenterology 1976;71:102–8.
29. Nayak NC, Dhar A, Sachdeva R, Mittal A, Seth HN, Sudarsanam D, et al. Association of human hepatocellular carcinoma and cirrhosis with hepatitis B virus surface and core antigens in the liver. Int J Cancer 1977;20:643–54.
30. Omata M, Yokosuka O, Imazeki F, Ito Y, Mori J, Uchiumi K, et al. Correlation of hepatitis B virus DNA and antigens in the liver. A study in chronic liver disease. Gastroenterology 1987;92:192–6.
31. Ray MB, Desmet VJ, Bradburne AF, Desmyter J, Fevery J, De Groote J. Differential distribution of hepatitis B surface antigen and hepatitis B core antigen in the liver of hepatitis B patients. Gastroenterology 1976;71:462–7.
32. Suzuki K, Uchida T, Horiuchi R, Shikata T. Localization of hepatitis B surface and core antigens in human hepatocellular carcinoma by immunoperoxidase methods. Replication of complete virions of carcinoma cells. Cancer 1985;56:321–7.
33. Jilbert AR. Studies of the pathogenesis of hepadnavirus infection (PhD thesis). Adelaide: University of Adelaide; 1989.
34. Yang D, Alt E, Rogler CE. Coordinate expression of N-myc 2 and insulin-like growth factor II in precancerous altered hepatic foci in woodchuck hepatitis virus carriers. Cancer Res 1993;53:2020–7.
35. Jacob JR, Ascenzi MA, Roneker CA, Toshkov IA, Cote PJ, Gerin JL, et al. Hepatic expression of the woodchuck hepatitis virus X-antigen during acute and chronic infection and detection of a woodchuck hepatitis virus X-antigen antibody response. Hepatology 1997;26:1607–15.
36. Xu C, Yamamoto T, Zhou T, Aldrich CE, Frank K, Cullen JM, et al. The liver of woodchucks chronically infected with the woodchuck hepatitis virus contains foci of virus core antigen-negative hepatocytes with both altered and normal morphology. Virology 2007;359:283–4.
37. Li Y, Hacker H, Kopp-Schneider A, Protzer U, Bannasch P. Woodchuck hepatitis virus replication and antigen expression gradually decrease in preneoplastic hepatocellular lineages. J Hepatol 2002;37:478–85.
38. Radaeva S, Li Y, Hacker HJ, Burger V, Kopp-Schneider A, Bannasch P. Hepadnaviral hepatocarcinogenesis: in situ visualization of viral antigens, cytoplasmic compartmentation, enzymic patterns, and cellular proliferation in preneoplastic hepatocellular lineages in woodchucks. J Hepatol 2000;33:580–600.
39. Bill C, Summers J. Genomic DNA double-strand breaks are targets for hepadnaviral DNA integration. Proc Natl Acad Sci USA 2004;101:11135–40.
40. Gong SS, Jensen AD, Wang H, Rogler CE. Duck hepatitis B virus integrations in LMH chicken hepatoma cells: identification and characterization of new episomally derived integrations. J Virol 1995;69:8102–8.
41. Gong SS, Jensen AD, Rogler CE. Loss and acquisition of duck hepatitis B virus integrations in lineages of LMH-D2 chicken hepatoma cells. J Virol 1996;70:2000–7.
42. Gong SS, Jensen AD, Chang CJ, Rogler CE. Double-stranded linear duck hepatitis B virus (DHBV) stably integrates at a higher frequency than wild-type DHBV in LMH chicken hepatoma cells. J Virol 1999;73:1492–502.
43. Yang W, Summers J. Illegitimate replication of linear hepadnavirus DNA through nonhomologous recombination. J Virol 1995;69:4029–36.
44. Yang W, Summers J. Infection of ducklings with virus particles containing linear double-stranded duck hepatitis B virus DNA: illegitimate replication and reversion. J Virol 1998;72:8710–7.
45. Yang W, Summers J. Integration of hepadnavirus DNA in infected liver: evidence for a linear precursor. J Virol 1999;73:9710–7.
46. Mason WS, Jilbert AR, Summers J. Clonal expansion of hepatocytes during chronic woodchuck hepatitis virus infection. Proc Natl Acad Sci USA 2005;102:1139–44.
47. Beasley RP, Trepo C, Stevens CE, Szmuness W. The e antigen and vertical transmission of hepatitis B surface antigen. Am J Epidemiol 1977;105:94–8.
48. Chen HS, Kew MC, Hornbuckle WE, Tennant BC, Cote PJ, Gerin JL, et al. The precore gene of the woodchuck hepatitis virus genome is not essential for viral replication in the natural host. J Virol 1992;66:5682–4.
49. Garcia PD, Ou JH, Rutter WJ, Walter P. Targeting of the hepatitis B virus precore protein to the endoplasmic reticulum membrane: after signal peptide cleavage translocation can be aborted and the product released into the cytoplasm. J Cell Biol 1988;106:1093–104.
50. Ou J-H, Yeh C-T, Yen BS. Transport of hepatitis B virus precore protein into the nucleus after cleavage of its signal sequence. J Virol 1989;63:5238–43.
51. Schlicht HJ, Salfeld J, Schaller H. The duck hepatitis B virus pre-C region encodes a signal sequence which is essential for synthesis and secretion of processed core proteins but not for virus formation. J Virol 1987;61:3701–9.

52. Pult I, Abbott N, Zhang YY, Summers JW. Frequency of spontaneous mutations in an avian hepadnavirus infection. J Virol 2001;75:9623–32.
53. Litwin S, Toll E, Jilbert AR, Mason WS. The competing roles of virus replication and hepatocyte death rates in the emergence of drug-resistant mutants: theoretical considerations. J Clin Virol 2005;34(Suppl 1):S96–107.
54. Yeh CT, Lin WP, Hsu CW, Chang ML, Lin SM, Sheen IS. Emergence and takeover of precore-stop mutant prior to exacerbation of e antigen-negative chronic hepatitis B after withdrawal of lamivudine therapy. J Med Virol 2006;78:906–10.
55. Dandri M, Burda MR, Will H, Petersen J. Increased hepatocyte turnover and inhibition of woodchuck hepatitis B virus replication by adefovir in vitro do not lead to reduction of the closed circular DNA. Hepatology 2000;32:139–46.
56. Protzer U, Nassal M, Chiang PW, Kirschfink M, Schaller H. Interferon gene transfer by a hepatitis B virus vector efficiently suppresses wild-type virus infection. Proc Natl Acad Sci USA 1999;96:10818–23.
57. Zhang YY, Zhang BH, Theele D, Litwin S, Toll E, Summers J. Single-cell analysis of covalently closed circular DNA copy numbers in a hepadnavirus-infected liver. Proc Natl Acad Sci USA 2003;100:12372–7.
58. Lenhoff RJ, Luscombe CA, Summers J. Acute liver injury following infection with a cytopathic strain of duck hepatitis B virus. Hepatology 1999;29:563–71.
59. Lenhoff RJ, Luscombe CA, Summers J. Competition in vivo between a cytopathic variant and a wild-type duck hepatitis B virus. Virology 1998;251:85–95.
60. Summers J, Smith PM, Huang MJ, Yu MS. Morphogenetic and regulatory effects of mutations in the envelope proteins of an avian hepadnavirus. J Virol 1991;65:1310–7.
61. Zhang YY, Summers J. Enrichment of a precore-minus mutant of duck hepatitis B virus in experimental mixed infections. J Virol 1999;73:3616–22.
62. Zhang Y-Y, Summers J. Low dynamic state of a viral competition in a chronic avian hepadnavirus infection. J Virol 2000; 74:5257–65.