Medical Hypotheses (2005) **65,** 509–520

ELSEVIER

medical hypotheses

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Multiple sclerosis and hepatitis B vaccination: Could minute contamination of the vaccine by partial Hepatitis B virus polymerase play a role through molecular mimicry?

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Received 24 January 2005; accepted 17 February 2005

Summary Reports of multiple sclerosis developing after hepatitis B vaccination have led to the concern that this vaccine might be a cause of multiple sclerosis in previously healthy subjects. Some articles evidenced that minor Hepatitis B virus (HBV) polymerase proteins could be produced by alternative transcriptional or translational strategies. Their detection is very difficult because they are in minute concentration and probably enzymatically inactive, however, it was shown that they could be exposed on the outside of the virus particles and also be immunogenic. In addition, HBV polymerase shares significant amino acid similarities with the human myelin basic protein. We hypothesise that some of the apparent adverse reactions to the vaccine could be due to a process called of molecular mimicry, the HBV polymerase, which could be a contaminant in the recombinant or plasma-derived vaccines, could act as autoantigens and induce autoimmune demyelinating diseases such as multiple sclerosis.

Introduction

There is a large volume of literature available on the Hepatitis B virus (HBV) and hepatitis B disease [1]. HBV infection is a major cause of acute and chronic hepatitis, cirrhosis, and hepatocellular carcinoma. Two billion of the six billion people alive today show evidence of past or current infection with this virus and ~350–400 million people are chronic carriers of HBV. HBV is an enveloped, partly double-stranded DNA virus containing a genome of approximately 3200 base pairs. The (complete) minus strand of the virus contains four overlapping open reading frames (ORFs): S, for the surface or envelope gene which is completely overlapped by the polymerase gene; C, for the core gene; X, for the regulatory X gene; and P, for the polymerase gene (Fig. 1) [1]. The HBV polymerase (HBV-pol) contains four domains, the terminal protein and spacer domains which are unique to the hepadnavirus polymerases and the reverse transcriptase and RNase H domains which contain the

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0306-9877/$ - see front matter
doi:10.1016/j.mehy.2005.02.044

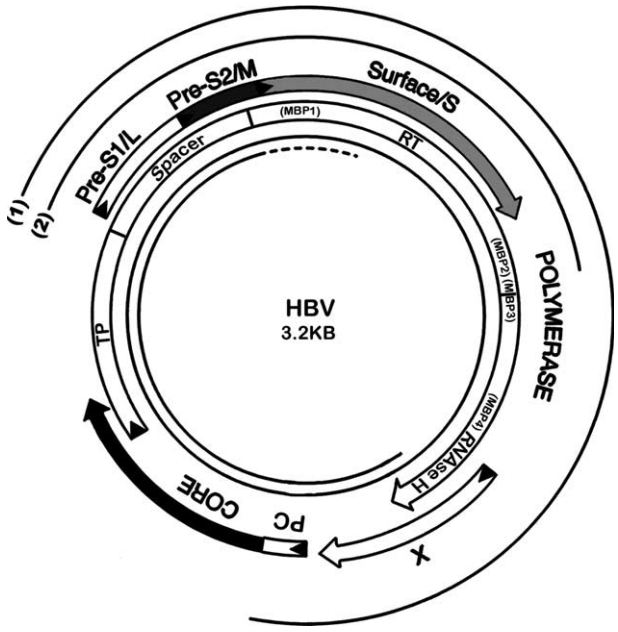

Figure 1 The overlapping genome of HBV. The inner circle depicts the complete minus-strand DNA (solid sphere) and the solid half sphere depicts the incomplete plus-strand DNA. The four protein-coding regions are shown between the inner and outer partial circles. They include the precore (PC) and core genes, the polymerase gene, and the X gene. The envelope genes pre-S1 (L), pre-S2 (M), and surface (S) are completely overlapped by the polymerase gene. The polymerase gene includes the terminal protein (TP), spacer, reverse transcriptase (RT) and the RNase H domains. The positions of the regions of polymerase (MBP1, MBP2, MBP3 (for MP3 and MBP3′) and MBP4) which share similarity with human basic myelin protein are indicated in the polymerase ORF (see Fig. 2). In addition, the approximate length of the HBV genome fragments used to produce the major recombinant vaccines are indicated by the number (1) for GenHevac® vaccine and (2) for Engerix-B® and Recombivax HB® vaccines.

conserved regions A through F and the two known enzymatic active sites [2]. HBV express three envelope components called S, M, and L. S (226 amino acids long), the smallest, defines the S domain. The sequences of all three Ag-proteins are coterminally identical but differ N-terminally due to translation initiation at three different in-phase start AUG-codons. The extra domain of M is known as pre-S2, while the domain unique to L is called pre-S1 (Fig. 1). Besides infectious virions (Dane particles), HBV-infected hepatocytes produce non-infectious subviral particles as 22-nm spheres and tubular forms [termed hepatitis B surface antigen (HBsAg)]. HBsAg particles contain viral-encoded membrane proteins (S, M and L) and host-cell-derived lipids [1]. HBsAg is conventionally classified into serotypes (*adw*, *ayw*, *adr*, and *ayr*) and height genotypes (A—H) of HBV have been identified which show some distinct geographic distributions [3]. As there is no effective treatment for this disease, the prevention of hepatitis B requires the availability of large quantities of effective, safe and affordable hepatitis B vaccine. During the 1970s, HBsAg purified from the plasma of healthy HBV carriers, was used to develop several hepatitis B vaccines. These vaccines were composed of non-infectious 22 nm HBsAg subviral particles purified by physicochemical methods. The first plasma-derived hepatitis B vaccine has been used in 1982. The drawbacks of the first generation of plasma-derived vaccines (relatively poor acceptance, relatively high cost, and limited availability) have led to the search for alternative means of producing hepatitis B vaccines. In the mid-1980s recombinant DNA technology was used to express HBsAg. The new technologies offer manufacturers a shorter production cycle, batch-to-batch consistency, and continuous supply of material, allowing the replacing of plasma-derived vaccines available on the market [4]. Plasma-derived and recombinant vaccines were shown to be immunogenic and effective in preventing hepatitis B virus infection in individuals at high risk for acquiring infection. The hepatitis B vaccine is over 95% effective in preventing chronic hepatitis B infection, and it is the first vaccine against a major human cancer, it has also been considered one of the safest vaccines ever produced [5]. However, a number of individuals who received the recombinant HBV have reported adverse events, some serious. Their medical complaints cover a spectrum of autoimmune and nervous system disorders, including central nervous system demyelination that resemble multiple sclerosis (MS) [6—9].

In the past 30 years many medical authorities have discussed and warned about possible neurological complications associated with hepatitis B vaccines, partly in recognition of the extrahepatic manifestations of the HBV disease and their possible relation to the HBsAg used in the vaccine [7,10]. Both activation of previously existing MS and the appearance of new CNS demyelination consistent with MS have been reported following hepatitis B vaccination. In addition, both syndromes have been reported in close temporal relationship following the administration of hepatitis B vaccine [11—13]. The temporal relationship between vaccination and the development of neurologic syndromes is an important consideration in the establishment of a possible causal relationship. In addition, few studies showed an increase of the relative risk of post-vaccination demyelinating

disorders, two French studies found about a 1.5-fold increase in the risk of a first episode of CNS demyelination during the 2 months following hepatitis B vaccination [14], in addition, the authors of a recent study estimated that immunization against hepatitis B was associated with a 3-fold increase in the incidence of MS within the 3 years following vaccination [15]. In spite of the fact that most of the studies conclude that association between the vaccine and MS is null, some findings are consistent with the hypothesis that immunization with hepatitis B vaccine could be associated with an increased risk of MS, and challenge the idea that the relation between hepatitis B vaccination and risk of MS is well understood.

On that account the characteristics of the hepatitis B vaccines were carefully analyzed. The plasma-derived vaccines are free of detectable nucleic acid [5]. Recombinant technology for hepatitis B vaccine involves the insertion of segments of the HBV genome which encode HBsAg into a plasmid in yeast or mammalian (Chinese hamster ovary, CHO) cells, thus allowing for the expression HBsAg. The desired protein(s) is(are) expressed and assembled into 22 nm antigenic particles [4,5]. The two major yeast-derived hepatitis B vaccines that are licensed in most countries are Engerix-B® (SmithKline Beecham) and Recombivax HB® (Merck & Co.) [5,16,17]. Both recombinant products are composed of the S antigen (subtype adw_2), without the pre-S regions. The HBsAg in the vaccine is the 22 nm subviral particle, composed of a non-glycosylated 226 amino acid polypeptide. The GenHevac B® (Pasteur Mérieux) vaccine is derived from transfected CHO cells and contains 22 nm of HBsAg particles, including both preS2 and S envelope proteins (subtype ayw_2) [4,18]. In these three recombinant vaccines the residual protein and DNA levels are much lower than the residual levels recommended by OMS. In addition, these vaccines could also contain various other contaminants like thiomersal, alum, formaldehyde, yeast- or mammalian derived lipids, but because neurological complications have been observed after both hepatitis B infection and vaccination, these different contaminants do not appear implicated.

MS is a mysterious disease which differs greatly from the liver ailments induced by HBV [19]. It is known as a ''chronic demyelinating inflammatory disease'' because it is accompanied by destruction of myelin, or the sheath or coating around nerve fibres. There are numerous reports that suggest viral infection may precede autoimmune diseases such as MS [20,21]. Mechanisms by which viruses may play a role in the development of autoreactive immune responses include: polyclonal activation of B and/or T cells, molecular mimicry, viral infection of immune cells, exposure of sequestered antigens, or altered host cell expression (''neoantigen or altered self'') in virus infected host cells [22]. The theory that molecular mimicry between viral and self antigens could, in some instances, initiate autoimmunity has gained increasing acceptance in the past few years and it is accepted that MS would be truly autoimmune and normal myelin antigens would be the target of a T-cell-mediated attack possibly initiated by molecular mimicry and perpetuated by an aberrant immune response, molecular mimicry, implying some level of analogy between a self Ag and an infectious agent [22–25]. $CD4^+$ T cells specific for myelin antigens are found in the blood of MS patients, and studies on antigen recognition showed that $CD4^+$ autoreactive, myelin-specific T cells from MS patients cross-react with peptides derived from bacterial or viral proteins [24,26]. Overall, these studies suggest that the exposure to pathogens may stimulate the self-reactive T cell repertoire such that it may trigger or exacerbate autoimmunity, this mechanism can operate for both Ab- and T cell-mediated autoimmune diseases [27,28]. Analysis of T-cell receptor recognition of major histocompatibility complex-bound peptides in light of this structural information demonstrated that specificity was typically confined to a small number of peptide side chains. Two groups have demonstrated that a viral peptide with homology at just 4–5 amino acids with the myelin basic protein (MBP) $peptide_{1-11}$ can induce clinical signs of experimental autoimmune encephalomyelitis (EAE) in susceptible mices, and only 2–4 native MBP residues were required for activation of MBP-specific T cells [28,29]. Interestingly, the concept of molecular mimicry was first tested in an experimental animal model with a HBV-pol peptide in which six amino acids were identical to the encephalitogenic region of rabbit MBP [23]. Peripheral blood leukocytes of rabbits immunized with HBV-pol proliferated in vitro to the viral peptide and intact MBP. Furthermore, animals injected with an eight- or ten-amino acid peptide from HBV-pol showed an antibody response against HBV-pol and native MBP. However, while the authors of this study were able to demonstrate inflammatory infiltrates in the CNS of animals immunized with HBV-pol, they were unable to elicit clinical EAE [23]. This study of the HBV-pol was used as the basis for the molecular mimicry model of autoimmune disease. However, as it is acknowledge that all the hepatitis B vaccines do not contain the HBV-pol, there would be therefore no risk that such a mechanism could cause MS after hepatitis B vaccination. It would be more logical to think that HBsAg

would be to imply in the adverse effects and not HBV-pol.

If an infectious agent has been associated with a particular adverse health outcome, the possibility exists that a vaccine against that agent could have a similar effect. As early as 1977, London first reported that autoimmune disease was caused by circulating immune complexes caused by (HBV) viral antibody association [30]. The primary effect of HBV infection is hepatic, but occasional extrahepatic manifestations. Acute or chronic active hepatitis B infections have been known, although rarely, to be associated with autoimmunity, demyelination and other polyneuropathies, resembling the adverse reactions reported as a consequence of the vaccine, discussions and case reports regarding autoimmunity occurring with the hepatitis B infection have been presented by some reviews [31–33]. However, the number of anecdotal reports which mention an association between HBV infection and central or peripheral neurological manifestations are not a prominent feature of HBV infection, and the majority of these data do not indicate a causal association between hepatitis B virus and autoimmunity diseases [34–36]. In addition, antiviral treatment, like interferon α, could be implicated in the development of several autoantibodies as well as in the development or exacerbation of various autoimmune disorders [37,38]. However, the majority of the side effects reported to the hepatitis B vaccines are the same or similar to those (rarely) reported as extrahepatic manifestations of the virus itself, their temporal relationship to hepatitis B vaccination, and the possible immune complex mechanism suggests a possible etiologic link with hepatitis B vaccine [7]. Both the plasma-derived and the recombinant vaccines contain the HBsAg and this protein could stimulate the immune system so as to cause life threatening autoimmune conditions, similar to those manifesting in people suffering from HBV. As early as 1975, in an article entitled ''Hepatitis Vaccine: A note of caution,'' Zuckerman warned that autoimmunity could follow the administration of the hepatitis B vaccine because the disease, itself, involved autoimmunity and he suggested, ''careful assessment of all vaccine effects on the immune system'' [39]. In addition, as late as 1988, Hilleman [40], considered the world's leading vaccine developer, warned ''the message from the hypothetical hepatitis B example is that the administration of antigens or monoclonal antibodies that directly or indirectly raise antibodies that attach to host cell receptors may carry large liabilities even though they might provide a convenient means for preventing viral access to host cells...antibodies attached to cell receptors may invite the same kinds of adverse response that are believed to be responsible for a variety of autoimmune disorders.'' As it is known, that self peptides from human myelin proteins can induce autoreactive $CD8^+$ Cytotoxic T cells and that these T cells produce cytokines thought to be important in mediating demyelinating disease [41]; that proliferative and cytolytic $CD4^+$ T cells from MS patients recognize myelin proteins [42], and that viral peptides activate human T cell clones specific for MBPs [24], studies to evaluate the similarities and the identities of peptide sequences of the HBsAg and myelin protein peptides thought to be important in demyelinating disease have been initiated. As other authors, we have searched the major protein databases for local sequence similarities between the HBsAg and various variants of human myelin components, but all these analyses do not show strikingly similar regions between HBsAg peptides and the myelin proteins. However, as a single T cell receptor can recognize quite distinct but structurally related peptides [24], further experiments which carry out more detailed structural analysis to identify peptide motifs are necessary, particularly since, a recent report describes the onset of clinically definite MS after hepatitis B vaccination, a T cell line specific for HB antigen that cross-reacts with PLP-derived peptide; the cell line was closed from $DR2^+$ patient who developed MS after HB vaccination [43]. The significance of these very preliminary findings needs to be confirmed but this suggests that the mechanism of molecular mimicry warrants further investigation. Although several studies reported the specificity of intrathecal oligoclonal bands against specific pathogens in other CNS diseases, no antibody against HBsAg has been identified in MS. However, recurrent demyelinating syndromes have been reported in patients with high titers of HBsAg [34,35].

The hypothesis

The similarity between serious adverse reactions to hepatitis B vaccines and the extrahepatic manifestations of hepatitis B infection, their temporal relationship to hepatitis B vaccination, and the possible immune complex mechanism suggest a possible etiologic link with hepatitis B vaccine. As computer analyses do not show strikingly similar regions between HBsAg peptides and the myelin

proteins, we hypothesise that trace amount of partial or fusion HBV polymerase protein could be co-purified with HBsAg during the manufacturing processes and that this protein could trigger the immunologic processes that lead to MS by a molecular mimicry between HBV-pol and myelin.

Evaluation of the hypothesis: Is there a causal link between HBV polymerase and post-vaccination demyelinating disorders?

Obviousness of alternative processes of HBV polymerase transcription and translation

In the recombinant vaccines, the plasmids used for the transfection do not contain the entire *pol* gene. For example, in the Genhevac B® vaccine, the plasmid used for the transfection of CHO DHFR-cells carries the HBV 2.3-kpb DNA fragment including the pre-S region, the S domain and the HBsAg mRNA polyadenylylation site [18,44]. In addition, in Recombivax HB® and Engerix-B® recombinant vaccines, yeast cells have been transfected with plasmids which contains, as part of HBV genome, only the S domain with short HBV flanking regions [5]. It is well known that viruses have developed a variety of strategies to maximize the number of proteins encoded by their size-limited genomes. Many of these strategies can involve control of gene expression at the transcriptional level and/or alternatives to conventional translational processes. For example, additional proteins may arise from the use of secondary translation initiation sites within an mRNA to access another ORF [45]. We have already underlined that the three envelope proteins (L, M and S) are the result of alternative translation initiations from the same ORF. Similarly, if C is translated by standard mechanisms from the 5′ ORF on the pregenomic RNA, the *pol* ORF overlaps the 3′ half of the C ORF, yet despite this unusual genetic organization, P is translated by de novo initiation at the first AUG of the *pol* ORF [46]. However, the precise mechanism for HBV-pol translation is not completely known, although a leaky scanning mechanism or termination, backwards scanning, and reinitiation have been postulated [2]. The *pol* gene of the hepadnaviruses appears to encode a major protein with a molecular size on the order of 90 kDa but numerous articles evidenced that, in this virus family, various HBV-pol variants could be synthetised in vivo. Some DNA polymerase activity gel studies on disrupted HBV viral particles have identified both ca. 90- to 110-kDa and ca. 60- to 70-kDa activities, with most of the activity at 60—70 kDa, a reverse transcriptase activity was also found at ca. 40—50 kDa [47]. As demonstrated for S gene, some minor P proteins could be initiated at various internal AUGs, this mechanism has been shown to occur for translation of the HBV-pol protein [48].

In addition, more of the major HBV unspliced transcripts which have been unequivocally demonstrated, several pregenomic RNA-derived spliced transcripts of HBV have been found in liver tissues or in hepatoma cell lines transfected with cloned HBV DNA. Furthermore, the majority of these spliced RNAs contain ORFs encoding various P- and S-related proteins but only a few number of their protein products has been reported. For example, a 10-kDa protein encoded by a singly spliced RNA was reported to be expressed in HBV-infected livers and this HBV splice-generated protein corresponds to the fusion of a part of the viral polymerase and a new ORF that is created by the splicing event [49]. In addition, a 43-kDa HBV polymerase/surface fusion protein encoded by a spliced RNA was also reported [50]. This glycoprotein was demonstrated to be a structural protein of Dane particles and 22-nm subviral particles and a HBV-pol epitope of this protein may be exposed on the surface of these particles. Structurally, this protein is similar to the L protein with the exception that the N-terminus of the LS protein is replaced by the first 47 amino acids of the N-terminus of the P protein. Sequence alignment showed the presence of consensus splice donor and acceptor sites at junctions which were conserved among various subtypes of HBV. Interestingly, in woodchuck hepatitis virus nucleocapsid particles various HBV-pol polypeptides have been found which have different amino-terminal but the same carboxy-terminal sequences [51]. In addition, using duck hepatitis B virus mutants, Wu et al., [52] have evidenced that less-than-full-length *pol* gene products are functional in synthesis of this virus.

This short bibliography analysis suggests that, in spite of the fact that the *pol* gene is not entire in the plasmids used to produce recombinant vaccines, truncated or fusionned HBV-pol protein could be synthesised by various processes including, for example, downstream translational initiation events and splicing. Similarly, contaminant

proteins including partial HBV-pol could be co-purified with the 22 nm HBsAg subviral particles in the plasma-derived vaccines.

Some antibodies against HBV polymerase are autoantibodies

Numerous studies have evidenced that the immune response toward the polymerase gene product is induced during acute and chronic HBV infection [53–55]. In addition, some of them are autoantibodies. For example, autoantibodies to smooth muscle and nuclear components are commonly detected in patients with chronic HBV infection [56]. Structural similarities of HBV-pol and four human nuclear and two smooth muscle proteins have been evidenced and three of these proteins share in common an amino acid sequence motif with HBV-pol$_{99-118}$ [57]. Since one of these motif-containing peptides is derived from the nuclear autoantigen PM-sclerosis (PM-scl Ag$_{761-780}$), it is possible that anti-nuclear Ab and anti-smooth muscle Ab may arise, at least in part, as a consequence of immunological cross-reactivity with a single determinant of HBV-pol$_{99-118}$ [57]. Double reactivity to HBV-pol peptide and self homologue was observed almost exclusively in patients with chronic HBV infection, providing a clear indication that these Abs are generated specifically as a consequence of HBV infection. In addition, HBV-pol shares amino acid sequence similarities with an uveitopathogenic peptide (peptide M) of retinal S-antigen (S-ag), a major autoantigen in experimental autoimmune uveitis (EAU) [58]. EAU was induced in Lewis rats with the synthetic peptide, corresponding to the amino sequence of HBV-pol, containing five consecutive amino acids identical to peptide M in S-ag. Lymph node cells from rats immunised with either peptide M or the synthetic peptide spanning the viral sequence showed a significant degree of cross-reactivity. These immunological cross-reactivities between HBV-pol and homologous regions of human proteins introduce the intriguing possibility that the host anti-HBV-pol response may contribute to the generation of extrahepatic autoimmune diseases in chronic HBV infection through molecular mimicry.

Human myelin basic proteins share common amino acid motifs with HBV polymerase

As, there is no such close linear relationship between HBsAg and CNS antigens which play a role in the pathogenesis of MS, and since, in one hand in a rabbit model, molecular mimicry between the HBV-pol and an immunodominant region of MBP which is the major autoantigen in multiple sclerosis, gives rise to autoimmunity, illustrating the potential for HBV-pol to break tolerance and induce autoimmunity through a cross-reactive mechanism [23] and in another hand, that numerous studies let suppose that partial or fusion HBV-pol could be present in the vaccines in minute concentration, we have searched the major protein databases for local sequence similarities between the HBV-pol (belonging to three variants using for vaccine: *ayw*$_2$, *adw*$_2$ and *adr*) and various variants of human myelin components which are the most commonly investigated candidate CNS autoantigens (MBP, proteolipoprotein (PLP), myelin-associated glycoprotein (MAG) and myelin oligodendrocyte glycoprotein (MOG)). The complete sequences of the HBV-pol were serially divided into 12 amino acid segments, each overlapping the preceding segment by six amino acids. The resulting set of 12 amino acid sequences was used to scan the PIR and SWISSPROT (Genetics Computer Group, Madison, WI) protein databases for sequence analogy with human myelin proteins using the FINDPATTERNS motif search algorithm. In our protein database search only similarity regions with a length higher than 3 residues between partial HBV-pol and myelin components have been researched. This analysis revealed that no closed similarity have been found with PLP, MAG and MOG, but on the other hand, various regions of MBP exhibit high local sequence similarity to the HBV-pol (Fig. 2). There are four isoforms of human MBP, arising from alternate exon splicing, and the major 18.5 kDa isoform which contains the 11 amino acids (region B) encoded by exon 5, but not the 26 amino acids of region A encoded by exon 2 [59,60] and three HBV-pol regions share four amino acid sequences with all the MBP isoforms. One alignment (MBP1) shares similarity between HBV-pol and a peptide from human MBP that bound with high affinity by the MS-associated HLA-DR2 molecule [61,62], in addition, the corresponding nucleotide sequence is overlapped with the Surface/S gene and is thus included in all the recombinant vaccine plasmids. As for MBP1, the MBP3 region contains dominant B cell epitopes [63]. More surprisingly, the MBP3′ domain shares a similarity sequence with the region A [60], which is not present in the common 18.5 kDa isoform. But, this region is contained in two other isoforms (21.5 and 20.2 kDa) which are expressed in the developing human CNS [60] and it has been suggested that they may also be expressed by remyelinating oligodendrocytes in MS lesions [64]. The MBP4 domain shares similarity with a MBP region which is not

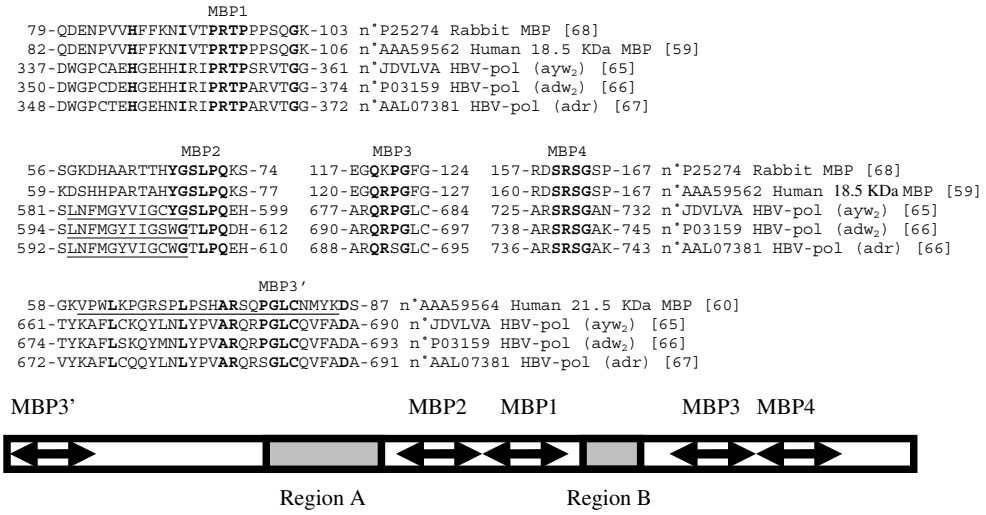

Figure 2 Amino acid sequence similarity between three subtypes (*ayw*, *adw* and *adr*) of HBV DNA polymerase (HBV-pol) and human and rabbit myelin basic proteins. Amino acids are in standard single letter code. Bold letters represent homologous amino acid residues. All the sequences are given with their GenBank accession numbers. The homologous sequences are respectively named MBP1, MBP2, MBP3, MBP3′ and MPB4, their respective positions in the HBV genome are indicated in the Fig. 1. The amino acid sequence of the major 18.5 kDa isoform of human MBP was used in MBP1, MBP2, MBP3 and MBP4 alignments and 21.5 kDa isoform for the MBP3′ similarity sequence. In the MBP2 and MBP3′ alignments, the underlined amino acid residues represent respectively the HBV-pol conserved region E [2] and the region A of human MBP [60], the rabbit MBP does not contain this domain. At the bottom of the figure, the positions of the HBV-pol sequences which share similarity with MBP are positioned on a schematic representation of the 21.5 kDa MBP isoform. The double arrows represent immunodominant epitopes of the whole MBP [62,63,70—74].

known as a target of auto-reactive T-cell responses and implicated in demyelinative disorders. In addition, the plasmids which bear only S domain with short flanking sequences do not contain MBP3, MBP3′ and MBP4 regions.

More interestingly, in subtype *ayw*$_2$ [65] a sequence similarity of 100% with the respective MBP peptide spanning 6 amino acid regions (MBP2) has been found. Alignment of homologous regions of the HBV-pol *ayw*$_2$ with the two other HBV subtypes used for vaccines (*adw*$_2$ [66] and *adr* [67]) show a less remarkable sequence conservation within homologous regions, but similar structural conformation does not have to be excluded. In another hand, in these three subtypes, the first residue 6-mer analogy region with MBP is the last residue of HBV-pol conserved region E. The *ayw*$_2$ subtype HBV-pol shares the six consecutive amino acids (-Y G S L P Q-) found as encephalitogenic site of rabbit MBP [68] in the Fujinami and Oldstone experiments [23]. In that study, rabbits immunized with HBV-pol peptide containing this six residues generated cross-reactive humoral and cellular immune responses to MBP and developed central nervous system lesions histologically similar to those seen in multiple sclerosis. In addition, it has been demonstrated that these six residues were a major EAE-inducing region of rat MBP in the Lewis rat and constitute the major encephalitogenic epitope of MBP for the Lewis rat [69].

Interestingly, the numerous variants of human MBP are aligned mainly with HBV-pol peptides corresponding to a region ranging between the medium of the reverse transcriptase domain and the medium of that of RNAse H. For example, alignment of this HBV-pol region with the entire major myelin isoform, divided into only five oligopeptides, exhibits a relatively high similarity (>17%) and identity (>34%) for non-homologous proteins. In addition, the majority of the similarity regions (excepted (MBP1)) are not in the overlapped HBV-pol sequence, this suggests that as this region is not locked, the mutations which allowed analogies with myelin could be accumulated, due to lower selection pressure on the non-overlapped regions.

In addition, the HBV-pol is regarded as a non-surface protein, which is generally the case, however, these amino-acid sequences similarities with myelin which probably implying molecular mimicry suggest that some epitopes of HBV-pol could be on the surface of the Dane and subviral particles and would play a role in the exhaust of the HBV to the immune system. Two strong arguments are in favour of this hypothesis. In one hand, it has been

found that HBV-pol and surface fusion proteins are present in some 22-nm HBsAg particles and that an epitope of these proteins may be exposed on the outside of these particles [50]. In another hand, this sequence similarity is probably not at random, because on the basis of needing five to six amino acids to induce a monoclonal antibody response, the probability of 20 amino acids occurring in six identical residues between two proteins is 20^6 or 1 in 128,000,000 [20], however, as the genetic code degenerated, there exists, for a given polypeptide, a set of synonymous sequences which would code the same polypeptide, in this case, the probability to have these six consecutive amino acids (-Y G S L P Q-) would be rather 1 in 2,982,600.

In summary, these similarities, whose majority are in regions which are known as immunodominant epitopes T- and/or B-cell and contained dominant epitopes in some MS patients [62,63,70—74], introduce the intriguing possibility that the host anti-HBV-pol response may contribute to the generation of MS disease in vaccined people through molecular mimicry.

Analysis of the bibliography suggests that the number of adverse effects is higher after recombinant-vaccine injections than after plasma-derived-vaccine injections or during chronic diseases. We cannot exclude that the synthesis level of abnormal HBV-pol protein in transfected cells is higher than during the hepatitis disease. However, if the HBV-pol level in 22 nm particles is very low it is non-null, indeed, part of HBV-pol have been found in some of these particles [50]. As adverse reaction reports after plasma-derived vaccines that the same types of adverse reactions are being reported for the recombinant forms of the vaccine probably the same Ag could be implicated. For example, immunity to HBV-pol protein may possess the potential of inducing CNS demyelination in that there have been several patients reported in whom HB vaccination using plasma-derived vaccine was followed by autoimmune adverse effects [11,75].

Consequences of the hypothesis and discussion

Our hypothesis could be an explanation of the apparent adverse reactions to the HBV vaccines, by a process called of molecular mimicry, the HBV-pol protein, which could be a contaminant in recombinant or plasma-derived vaccines, could provoke an autoimmune attack on a similar protein found in the nerves or other tissues. These minor HBV-pol proteins could be initiated at various internal AUGs of the *pol* gene or by other processes including putative alternate splicings and this part of protein could be co-purified with the HBsAg proteins. We warned of molecular mimicry and the possible dangers associated with using genetically engineered hepatitis B vaccines containing polypeptide sequences that are present in human neurologic tissues, such as myelin. We hypothesize that through molecular mimicry, some HBV-pol polypeptides can act as autoantigens and induce autoimmune demyelinating diseases such as multiple sclerosis. Although our hypothesis requires further investigations, it suggests a series of studies that could be done on vaccines and on patients who developed complications after the hepatitis B vaccination.

No hepadnavirus-specific DNA polymerase activity has been found in the plasma-derived and recombinant vaccines. This failure may be due to technical reasons. This supports the possibility that partial polymerases are truly enzymatically inactive but they could be immunogenic. Antibodies to the polymerase can be found in people infected with HBV [53—55]. However, if the HBV-pol were made only in the trace amounts, it would be hard to imagine how antibodies could routinely develop against it, especially as the polymerase is rarely exposed on the outside of the virus. It is necessary, in the future, in order to detect the minute amount of HBV-pol or its related proteins to develop highly sensitive methods.

In order to test whether the structural similarities of HBV-pol and human myelin proteins produce cross-reactivity, homologous peptides could be constructed and used them as targets in an ELISA for the sera of vaccined patients which exibit autoimmune adverse effects. The finding of cross-reactive Ab responses between HBV-pol peptides and homologous regions of human nuclear and smooth muscle proteins suggests that autoantibodies may have arisen as an inappropriate evolution of the anti-HBV-pol immune response, to include antigenically similar human proteins [57]. In our study only ''linear'' similarities have been investigated but it may still be conceivable that HBV-pol-derived peptides could induce a humeral autoimmune response may still be conceivable, as antibodies recognize both conformational and linear epitopes. It is clear that detailed molecular and biochemical analyses, including analyses of the tertiary structure, need to be carried out to identify specific peptides which might be acting as molecular mimics. All the peptide products, and not only HBV-pol, deduced from the HBV DNA sequence for each of the three ORFs should be

tested for the extent of their polypeptide analogy with all human proteins. As suggested by Wucherpfennig and Strominger [24], genetic modifications of viral vaccines that eliminate proven mimicry epitopes could make viral vaccines safer and reduce the frequency of post-vaccinal encephalomyelitis, if significant identity/similarity were to be demonstrated, the offending polypeptides could be removed from the vaccines or synthetic vaccines could be produced without them. Some proposals for changes are presented in the Fig. 3. Upstream the two regions which share similarity to MBP, there are some putative start codons in the *pol* ORF in all the HBV variants, even if these codons are in a poor context for translational initiation [45], systematic mutagenesis of these putative initiation codons in the plasmid constructs could reduce the risk of synthesis of a truncated HBV-pol protein. In order to make impossible the reinitiation at a normally silent internal start codon in the *pol* ORF, some ATG in this ORF could be mutated in another codon. This type of mutation is relatively easy because the S ORF is +1 than the *pol* ORF, the first nucleotides in the *pol* ORF codons are the third of the S ORF codons and due to the degeneration of the genetic code, mutations of ATG to BTG (B being C or G or T) generally generated silent mutations in S ORF. An example of a mutation which abolishes a putative start codon upstream from the regions homologous to MBP and which did not alter the amino acid sequence of envelope protein is given in the Fig. 3. As we cannot exclude totally the production of the env-pol fusion protein, it is also possible to generate stop codon in the *pol* ORF upstream or in the regions homologous to MBP. Three examples of lethal missense mutations of the *pol* ORF are suggested (Fig. 3).

HLA patterns of vaccinated people might be a contributing factor. The HLA patterns of experimental animals have been shown to influence their susceptibility to experimental demyelinating diseases [76]. Although the causality between vaccination against hepatitis B and the onset of demyelinating syndrome had not been proven, it is suggestive that patients with adverse effects had A3, B7 and DR2 HLA haplotypes, which are associated with an increased risk of developing MS [77,78]. In the future, like that was already suggested [12,24], the HLA patterns and the percentage of their T-cells that exhibit antimyelin activity of the individuals who have been reported to autoimmune adverse effects could be determinated to ascertain if host factors are partially causative of the complication. In addition, analysis of TCR cross-reactivity for T-cell clones activated by HLA/MBP peptide complex could provide insights into disease mechanisms in MS [79].

In addition, the ''principle of precaution'' should also apply to future therapeutic strategies like DNA vaccines, virus-like particles or use of HBV-pol as vaccine. Virus-like particles structurally mimic the viral capsid and have therefore been extensively, and quite successfully, used as vaccine and viral serology reagents. The ability of virus-like particles to include nucleic acids or small molecules has also made them novel vessels for gene

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710  GGCTTTCCCCCACTGTTTGGCTTTCAGTTATATGGATGATGTGGTATTGGGGGCCAAGTCTGTACAGCAT
P-529  A  F  P  H  C  L  A  F  S  Y  M  D  D  V  V  L  G  A  K  S  V  Q  H
S-349   L  S  P  T  V  W  L  S  V  I  W  M  M  W  Y  W  G  P  S  L  Y  S  I
Suggestions of mutation:          T/C                    T
Results after the mutations in P: -  L  -  -  -  -  -  -  - Stop

780  CTTGAGTCCCTTTTTACCGCTGTTACCAATTTTCTTTTGTCTTTGGGTATACATTTAAACCCTAACAAA
P-552 L  E  S  L  F  T  A  V  T  N  F  L  L  S  L  G  I  H  L  N  P  N  K
S-372  L  S  P  F  L  P  L  L  P  I  F  F  C  L  W  V  Y  I  Stop

849   ACAAAGAGATGGGGTTACTCTCTAAATTTTATGGGTTATGTCATTGGATGTTATGGGTCCTTGCCACAA
P-575  T  K  R  W  G  Y  S  L  N  F  M  G  Y  V  I  G  C  Y  G  S  L  P  Q
Suggestions of mutation:                                   T     A
Results after the mutations in P: -  -  -  -  -  -  -  -  -  -Stop- -Stop
```

Figure 3 Partial nucleotide sequence of a fragment of HBV (subtype *ayw*$_2$) corresponding to the end of the S gene. The sequence genome of the minus-strand is shown. The corresponding sequences of the S and HBV-pol proteins are indicated below. Nucleotides and amino acids are numbered (numbers at left) as in Galibert et al. [65]. In order to abolish one of the putative start codon and to introduce stop codons in *pol* ORF, some DNA substitutions are proposed and the nucleotide and amino acid consequences indicated below. For mutant sequences, only nucleotides and amino acids that differ from the wild type are listed. The mutated nucleotides are in bold letters, the *pol* codons implied are in italic letters and underlined, while the region which share obvious similarity with MBP is in bold letters and underlined. In all these nucleotide mutations, the S amino acid sequence is unchanged.

and drug delivery [80]. These recombinant particles which are purified from yeast after expression of the HBV S gene could contain partial HBV-pol protein as minute contaminant. In another hand, DNA-based vaccination appears as a particularly pertinent approach for chronic hepatitis B therapy, since viral proteins are expressed in the cells after transient in vivo transfection with plasmid DNA and subjected to the same post-translational modification as during viral infection leading to extremely potent stimulation of humoral and cellular responses [81]. However, in HBV DNA vaccines, the plasmids which carrying genes encoding various HBV proteins could induce the synthesis of partial HBV-pol proteins. In another hand, some authors have proposed to use HBV-pol as vaccine component [82], what will deserve as a preliminary to check our hypothesis.

In conclusion, as there is no effective treatment for the HBV disease and as vaccination has proved vital for prevention and eradication of hepatitis B in developed world, the aim of this present paper is only to stimulate research on the putative molecular mimicry between HBV-pol (and other HBV proteins) and human proteins.

Acknowledgement

We wish to thank Noëlle Moretta for help in preparing the figures.

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