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HEPATITIS B VIRUS PROTEINS ELICITING PROTECTIVE IMMUNITY

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The immune response to current hepatitis B vaccines appears to be qualitatively different from the response elicited during recovery from natural infection. Such disparate responses can probably be explained by the absence or under-representation of preS-and the nucleoprotein core-specific determinants in the vaccines. The incorporation of these determinants into future vaccines may improve their efficacy.

Introduction

Viral structural proteins essential in the strategy of virus replication and of host-to-host transmission are also targets for the host's immune response. The host's defence mechanisms can be directed against the virus itself as well as against virus-infected cells, and may be mediated by antibodies and by lymphocytes, i.e. specific cytotoxic T (T_c) lymphocytes.

It is generally accepted that antibodies neutralizing the infectivity of viruses are directed against components exposed on the surface of the virus. The mechanism of antibody-dependent virus neutralization is, for most viruses, unclear at present. A single virus protein may exhibit different classes of antigenic determinants. Antibodies bound to some of these determinants may have little or no effect on the virus life cycle, while antibodies bound to other sites on the same protein may neutralize virus infectivity[1]. Different viruses usually have different mechanisms of neutralization and the outcome of the process of neutralization of infectivity may depend on the host cell. Since neutralizing antibodies are one of the main arms of protective immunity against viruses, a rational design of optimal vaccines for any particular virus requires sufficient knowledge about the virus-neutralization process.

Recent results indicate that both the surface proteins of viruses, and internal viral components contribute to the host's protective immune response. Viral proteins which are inaccessible on the surface of intact virus particles can be exposed on the membrane of infected cells and can thus become targets of either specific antibodies or of T_c lymphocytes, which will contribute ultimately to the elimination of cells in which the virus replicates.

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The elicitation of virus-neutralizing antibodies requires the collaboration of three distinct cell types, all of which must recognize specific sites on the virus proteins. Only one of them, the B lymphocyte, produces antibodies and recognizes the same sites on the virus protein (B-cell epitopes) as do the antibodies. The other two cell types are: (i) accessory (A) cells; and (ii) helper T(T_h) lymphocytes. When A cells present virus antigens associated with cell membrane proteins (class II histocompatibility antigens) to $T_h$cells, the cells become activated to produce lymphokines essential for replication and differentiation of various cell lineages involved in the immune response. Sites on virus proteins recognized by T_h cells (T-cell epitopes) are usually distinct from B-cell epitopes. T_h cells may react with the protein containing the virus-neutralizing epitope(s) or may recognize another, possibly an internal virus protein which becomes exposed *in vivo* as the result of partial virus uncoating. Thus, the T/B-cell cooperation may be based on recognition, by the respective cells, of distinct viral proteins on the same virus particle. Such interaction is termed intermolecular/intrastructural[2,3] to be distinguished from T/B-cell collaboration based on recognition of T- and B-cell epitopes localized on the same protein molecule (intramolecular help).

The exact features of protective immunity against many medically important viruses remain largely undefined. This review will summarize the emerging knowledge concerning the role of distinct hepatits B virus (HBV) structural proteins in eliciting protective immunity.

Hepatitis B is one of the major viral diseases throughout the world. There are about 280 million chronic carriers of HBV, and an estimated 20 million new infections occur annually. A causal link between HBV infection and primary hepatocellular carcinoma has been established and about 1 million new cases of hepatoma occur throughout the world each year. The number of deaths resulting from hepatocellular carcinoma is estimated to be about 350 000 each year. The late sequelae of HBV infection, including hepatocellular carcinoma, are one of the major causes of death in countries with a high prevalence of HBV carriers. Therefore, the prevention of HBV infections and the possible eradication of hepatitis B are of essential importance.

A possible means of providing protective immunization was discovered, much before knowledge on structural features of HBV was attained, when it was found that sera from HBV carriers (rendered substantially less infectious by heating) protected recipients against HBV infection.[4] Subsequent studies revealed that the immunogen present in these sera corresponded to subviral particles (HBsAg) containing virus envelope proteins (mostly S-protein) (see Centrespread illustration IV; see below). HBsAg particles purified from sera of HBV carriers by different methods became the basis of first-generation hepatitis B vaccines. These vaccines are efficacious in preventing HBV infections in most healthy recipients and in a proportion (depending on the manufacturing procedure) of immunosuppressed recipients (i.e. haemodialysis patients).[5,6] There is a proportion of non-responders to the vaccines (depending on the type of population and the source of vaccine), [5,7] and substantial differences in the levels of antibody response between individuals have been observed. Many non-responders to the vaccine who later became infected, and subsequently recovered, developed a good antibody response to HBV env proteins, including S-protein (representing the major protein component of the vaccines).[5,7,8] To quote Hadler et al.[7]: '... the disparate non-response to vaccine, as compared with the normal response to natural infection, remains a mystery...'. The shrouds of mystery can be dissipated by scrutinizing the immune response to the immunogen present in the vaccine in comparison with the immune response elicited

by natural infection. Such scrutiny necessitates precise information concerning the antigenic composition of viral subunits used for vaccination, and of intact virus particles.

HBV proteins

A computer search for initiation and termination codons on putative RNA transcripts from both the L- and the S-strands of HBV DNA species belonging to different serological subtypes of the virus, read in the three possible reading frames, identified four open reading frames (ORFs) on the DNA L-strand (see Centrespread illustration I). Two of the ORFs were unequivocally assigned to two distinct structural components of HBV: the envelope (env) proteins, and the internal nucleo-capsid core protein (HBcAg), respectively. There are two initiation codons at which the translation of the core (C) gene product could start, and three initiation codons at which the translation of the env gene product could start. Therefore, gene C is subdivided into the pre-core and core regions and the env gene into the preS1, preS2 and S regions. Region X of the HBV DNA is also transcribed and translated. However, the presence, location and possible function of X-protein within the HBV virion has not been established. Region P codes for the DNA polymerase/reverse transcriptase present in HBV particles. Assignment of these gene products to specific sequences within region P has not been accomplished. There are additional structural proteins in the virion: a protein covalently-linked to the 5' end of the L-strand of HBV DNA; a protein kinase found within the nucleocapsid core; and possibly additional non-structural proteins found in HBV-infected cells,[8,9]. The coding sequences for these proteins have not been established yet.

The DNA polymerase/reverse transcriptase, the protein linked to HBV DNA, and the protein kinase are minor components of the virus and are therefore unlikely to play a prominent role in eliciting defence mechanisms in infected hosts. Therefore, the major immunogens expected to play a role in protective immunity correspond to products of the env gene and of gene C. To date, there is no evidence for a possible role for the product of region X in the immune response, although it cannot be excluded. In order to understand the roles of the env and C gene products in protective immunity, it is necessary to discuss their properties in greater detail.

Several distinct mRNAs (S-, M- and L-RNA; see below) hybridizing with the env gene have been isolated from HBV-infected cells or from cells transfected with recombinant HBV DNAs (or portions thereof). The 5' - and 3' ends of these mRNAs were determined by S1 nuclease mapping and by primer extension analysis.[8] All the mRNAs have co-terminal 3' ends, but differ in their 5' ends (Centrespread illustratior II). These mRNAs can encode three distinct protein products (S-, M- and L- protein) showing variations at their N-termini but having a common C-terminus (Centrespread illustration III, upper right).

Greater than genome-length RNA species with the same 3' - terminal end as the mRNAs mentioned before, appear to serve as messages for the C-gene products. However, much smaller subgenomic fragments of HBV DNA, upon transfection, can direct the synthesis of proteins encoded by the C-gene.[10, 11]Proteins corresponding to the entire ORF or to the ORF with the pre-core sequence deleted were both synthesized in the transfected cells. The protein with the deleted pre-core sequence corresponds to the major component of the HBV nucleocapsid.

HBV envelope proteins

Translation products of each of the S-, M- and L-m RNAs (Centrespread illustration II) were identified in HBV. Because of differences in glycosylation, a total of six distinct proteins (glycoproteins) were discerned (Centrespread illustration III).[8, 9, 12–14] The relative proportions of these distinct protein species differ between virus particles and subviral particles (~20 nm spherical forms and tubules (Centrespread illustration IV and cover, section A). P25/GP29 (S-protein) are the major components of HBsAg particles, GP33/GP36 (M-protein) are much less abundant, while P39/GP42 (L-protein) are the least prevalent. The higher molecular-weight components are usually more abundant in tubules than in the ~ 20 nm spherical particles. The composition of subviral particles may differ at distinct stages of HBV infection.[8, 12] Although present in low amounts in subviral particles, P39/GP42 are essential components of HBV, about 40-80 copies being present in a single virus particle.[12].

Primary sequence analyses of S-, M-, and L-protein, indicated that preS sequences, compared with S sequences, are highly hydrophilic, and have a high content of charged amino acid residues. [8,9] These properties indicated that preS 1 and preS2 sequences are exposed on the surface of HBV and play an important role in immunological recognition and in reactions with cell receptors. The absence of cysteine residues (involved in disulphide-bond formation) in the preS region also suggested that these sequences would be much easier to mimic with synthetic peptide analogues as compared with sequences corresponding to S-protein. Some of these predicted properties are also evident from the antigenic index algorithm (Centrespread illustration III), indicating a much higher frequency of potential antigenic sites within the preS1 + preS2 sequence than within the S sequence.

These predictions are in agreement with the experimental finding that the frequency of contiguous B-cell epitopes on the preS1 + preS2 sequences is higher than that on the S sequence (Centrespread illustration V). Similar conclusions apply to the occurrence of T-cell epitopes. However, it has to be recognized that the antigenicity and the immunogenicity of S-protein depend on the maintenance of disulphide bonds. Therefore, some of the S-protein antigenic determinants would not be mimicked easily by synthetic peptides. Interestingly, the B-cell epitopes are localized on segments of the sequence with predicted beta-turns. T-cell epitopes encompassed regions containing some predicted alpha-helices, in agreement with the postulated importance of amphiphilic helices in T-cell epitopes.[8].

The HBV binding site for hepatocyte receptors was localized between residues preS(21-47) within the preS1 sequence. Antisera to the synthetic peptide preS(21—47) inhibited the reaction between HBV and HepG2 hepatoma cells. Less efficient inhibition was also observed with the preS 2-specific antiserum anti-preS2(120-145) (anti-preS(120-153)), probably because of steric inhibition or conformational changes within the preS1 sequence elicited by this antibody (Centrespread illustration V). The location of the binding site for the hepatocyte receptor was further confirmed by experiments in which the uptake of gold-labelled HBV by HepG2 hepatoma cells was followed (cover, section F). The uptake of the particles was completely inhibited by a mixture of synthetic peptides (preS(21-47) and preS(120-153)).

Hepatitis B core antigen (HBcAg)

Recent results show that expression of the pre-core region is not required for synthesis of HBcAg.[10, 11] However, the pre-core region allows the core protein to

become associated with cytoplasmic membranes, the endoplasmic reticulum and probably with the cell membrane. Thus, the pre-core sequence is instrumental in the targeting of the core proteins to cellular membranes. This is in full agreement with earlier results indicating that HBV-specific T_c-lymphocytes from HBV-infected individuals are directed against HBcAg expressed on the surface of infected hepatocytes.[20]

Lessons for hepatitis B vaccine design

HBsAg spherical subviral particles (Centrespread illustration IV; cover, section A) isolated from serum of HBV carriers or prepared by recombinant DNA techniques represent the only immunogens present in hepatitis B vaccines. Such vaccines, as discussed before, have a lower content of preS1 and preS2 sequences than HBV particles have, and completely lack HBcAg. Some manufacturing procedures completely remove preS1 and preS2 sequences from the immunogen (Centre spread illustration IV).[8,14] Although S-protein alone is known to elicit protective immunity in a high proportion of healthy recipients,[5-7] and monoclonal antibodies directed against an appropriate segment of the S-protein are virus neutralizing,[21] other epitopes present in HBV, and eliciting protective immunity, are absent or under-represented in such vaccines. These include three types of epitopes.

Epitopes on the preS2 sequence

These epitopes (i) evoke an immune response in humans recovering from hepatitis B;[8, 14] (ii) elicit virus neutralizing antibodies; [15] (iii) elicit protective immunity (in chimpanzees immunized with preS2-specific synthetic peptides);[22, 23] (iv) are immunodominant; [8,13,14,16] and (v) have the potential to provide T_h-cell help for an anti-protein-S-antibody response and thus can circumvent the non-responsive status to S-protein through preS2-specific T_h-cell helper functions.[8,16,17]

Epitopes on the preS1 sequence

These epitopes (i) elicit antibodies preventing the interaction between HBV and hepatocytes[19] and are expected to be virus-neutralizing by means of virus attachment blockade by antibodies; (ii) evoke an immune response in humans recovering from hepatitis B;[8, 14] (iii) contain epitopes which are more immunogenic at the B-cell and T-cell levels than epitopes on the S-region;[8, 18] and (iv) can circumvent immunological non-responsiveness to both S-protein and the preS2 region through preS1-specific T_h-cell functions.[18].

Epitopes on HBcAg

These epitopes elicit protective immunity in chimpanzees.[24,25] It is known that antibodies to HBcAg (anti-HBc) are probably not protective since such antibodies are present in most HBV carriers, and do not prevent HBV infection in babies who acquired anti-HBc transplacentally from HBV carrier mothers. The protective immunity elicited by active immunization with HBcAg may be explained by elicitation of HBcAg-specific T_c-limphocytes contributing to the elimination of HBV infected cells; and by intermolecular/intrastructural help[2,3] provided by anti-HBc-specific T_h - cells to anti-env protein-specific B lymphocytes.

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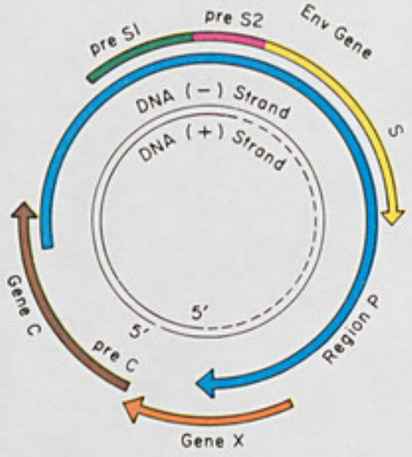
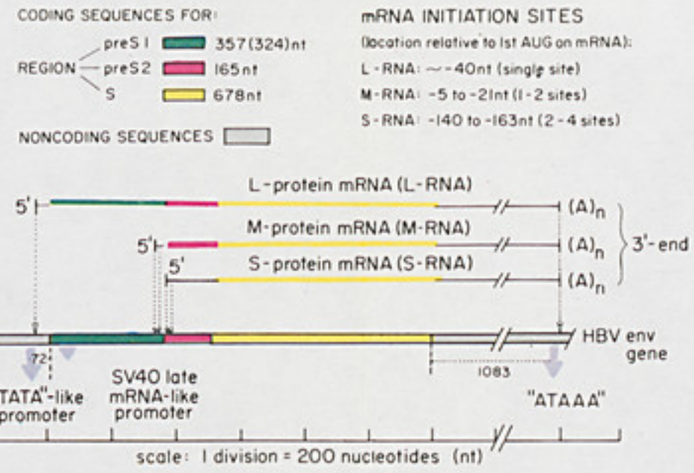


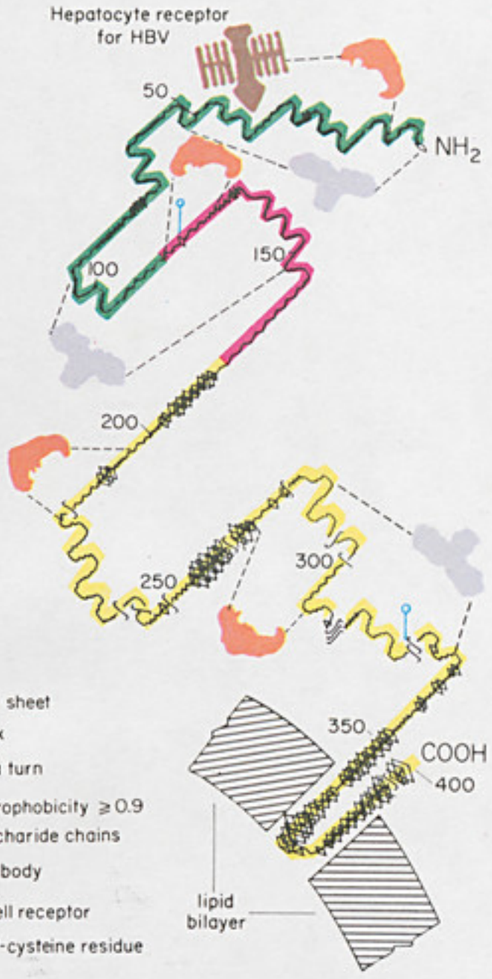
I

DIAGRAM I. Genetic map of HBV. The partially double-stranded DNA genome is drawn as the inner double circle; the innermost circle with a deleted region (indicated as a broken line) represents the DNA S- (small or plus) strand; the DNA L-(large or minus) strand has a nick located near the start of the open reading frame (ORF) for the core antigen (HBcAg) (gene C). The L-strand is the template for RNA transcripts. The ORFs are shown as broad arrows surrounding the genome.



II

DIAGRAM II. Schematic linear transcriptional map of the HBV envelope (env) gene. The positions of putative promoters controlling the transcription of the HBV env gene: (i) the 'TATA'-like promoter and (ii) a sequence resembling the late promoter of simian virus 40 (SV40), and the putative polyadenylation signal 'ATAAA' are indicated. The map is derived from results reviewed recently.[8]



V

DIAGRAM V. Predicted secondary structure of the HBV env gene product and identified regions containing B- and T-cell epitopes and the hepatocyte-receptor binding site. Locations of highly hydrophobic regions, of cysteine residues and of glycosylation sites are indicated. Based on studies with antisera to synthetic peptide analogues[8,9] encompassing the entire sequence, the only stretches of more than 10 AA not accessible to antibodies are between residues 256–268 and 360–385. The latter region is assumed to be embedded within a lipid bilayer, as shown. The other hydrophobic regions may also be associated with lipid components of the HBV envelope. The identification of B-cell epitopes was based on the recognition by human, rabbit or mouse anti-HBV (anti-HBs) of synthetic peptide analogues corresponding to segments of the L-protein sequence.[8,9,13–15] Epitopes made up of residues that are not contiguous in sequence may not be detected by this approach. The recognition of T-cell epitopes was based on identification of synthetic peptide analogues which induced proliferation of T-lymphocytes isolated from mice immunized with the native protein.[8,9,16–18] The hepatocyte-receptor binding sites were located by competitive binding assays using synthetic peptides and antisera to them as inhibitors of the HBV-hepatocyte attachment reaction.[19] The computer studies on the secondary structure were carried out by Dr John Devereux, University of Wisconsin.

IV

DIAGRAM IV. Schematic representation of individual structural subunits containing: (A) S-protein only; (B) M-protein, and (C) L-protein and of assembled HBsAg and HBV particles. The latter contain an internal nucleocapsid shown in the bottom split-open section.

III

DIAGRAM III. *Upper right: Schematic representation of HBV env proteins and their relatedness.* The open reading frame (ORF) on HBV DNA coding for HBV env proteins (see Diagram I) has the capacity to code for a protein consisting of 389-400 amino acids (AA), depending on the antigenic subtype of HBV. The 25 kD S-protein is derived from the *C*-terminal of the ORF and consists of 226 amino acids (AA). It exists in non-glycosylated (P25) and glycosylated (GP29) forms. The middle (M) protein (281 AA) contains the sequence of the S-protein with 55 additional *N*-terminal AA encoded by the preS2 region of HBV DNA, and occurs in two distinct glycosylated forms, GP33 and GP36. The large (L) protein (389 or 400 AA) contains the sequence of M-protein with 108 or 119 additional *N*-terminal AA encoded by the preS1 region of HBV DNA, and exists in a non-glycosylated (P39) and a glycosylaed (GP42) form.
Upper left: Schematic representation of results describing the separation of HBV env proteins by polyacrylamide gel electrophoresis (PAGE) under denaturing conditions. The proteins of HBV or of subviral HBsAg particles were detected either by a silver stain (the composition of the separated proteins is indicated by colour shading; for example GP42/P39 consists of preS1, preS2 and S sequences, indicated by green, red and yellow lines, respectively; the presence of the major protein of the viral nucleocapsid (HBcAg – the product of gene C; see Diagram I) is indicated by the brown band) or transferred to nitrocellulose membranes and subsequently detected by labelled antibodies specific for preS1 (green), preS2 (red) or S (yellow) sequences, respectively (only results with HBV are shown). Width and shading of bands is related to the quantitative proportions of the distinct env proteins.
Bottom: Antigenic index algorithm for L-protein.[8] This algorithm was designed to predict the location of antigenic sites (indicated by peaks above zero lines) from primary amino acid sequence data (Jameson and Wolf, in press).

Synthetic peptide analogues

Since it is currently impossible to obtain HBV in high yields, either from the plasma of HBV carriers or by cultivating the virus in tissue culture, the approaches for preparing vaccines containing all immunogens important in protective immunity have to rely either on recombinant DNA techniques or on synthetic peptides. Vaccines containing HBsAg particles rich in preS2 sequences, in addition to S-protein, are being developed.[8] However, constraints in the assembly of particles containing high levels of preS1 sequences[8] will probably make the recombinant DNA approach impracticable for the development of immunogens rich in preS1 sequences. For this reason, and also because of economic considerations, peptide synthesis might offer an attractive alternative approach to the design of hepatitis B immunogens.[8,26]

The preS2-specific peptides preS2(120-145) and preS(120-153), and the preS1-specific peptides preS(21-47) and preS(15-47), elicit high levels of antibodies recognizing native HBV particles (cover, sections B and C), and specifically recognize GP33/36 and P39/GP42 in Western blots (cover, sections D and E). As mentioned before, the anti-preS2-specific antibodies are virus neutralizing and the corresponding synthetic peptides elicit protective immunity. Similar experiments concerning synthetic analogues of the preS1 sequence are in progress. The role of an HBcAg-specific immune response in protective immunity, and the fine-specificity of this antibody response, is also amenable to experimental exploration using synthetic peptide analogues.

Conclusions

The disparate non-response to a hepatitis B vaccine, when compared with a good response to natural infection in some individuals, is less mysterious than it appears[7] and can probably be ascribed to the absence of essential epitopes in the vaccine described.[7] . Vaccination strategies considering preS-determinants, and possibly also HBcAg-determinants, should help to eradicate hepatitis B and the primary liver cancer associated with this disease.

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