THE CHALLENGE OF HEPATITIS B VIRUS

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Hepatitis B virus (HBV) is a very serious threat to public health in most of the developing countries of the world. It is estimated that around 300 million people worldwide are chronic carriers of this virus and will transmit the disease both vertically and horizontally. Infection by this virus may cause a wide range of clinical manifestations ranging from an asymptomatic infection to liver cirrhosis. Around 38% of the people who become chronic carriers eventually die of liver cancer (1). It has been found that around 50% of all patients with liver cancer and chronic carriers of HBV. Among the carriers, the risk of contracting hepatocellular carcinoma is 223 times higher than that of non-carriers (2). This makes HBV the most potent carcinogen in man (1). These data and other observations, clearly suggest that a significant number of liver cancers in the world are caused by HBV infection. In some cases, the infections are asymptomatic and any infection can only be confirmed by the presence of HBV genome, integrated in the host cellular DNA (3). Although the disease is known for a long time, like many other viral diseases, no cure for HBV infection has yet been found. That left the medical community with only one choice; namely, to prevent the spread of the disease, which has prompted vigorous research activity to produce a successful and economical vaccine against HBV. This review will concentrate on the disease caused by the virus, some interesting aspects of the virus itself, and the various approaches taken to make a vaccine against it.

DISEASE

The term "hepatitis" means inflamation of the liver, which may be caused by drugs, toxin, bacteria, excess alcohol intake and, finally, virus. Among the viruses which cause hepatitis, Hepatitis A, B and Delta are the most common (4). Cytomegalo virus and Epstein Barr virus also may cause hepatitis. There is another group of viruses called "NANB" whose identity has not yet been established, that are also linked to causing hepatitis. Since there is no direct serological test available for NANB viruses, those viral hepatitis infections that are caused neither by Hepatitis A nor by Hepatitis B are called NANB (non-A, non-B) and the hepatitis diagnosis is based on the principle of exclusion. Of all the cases reported in the United States, around 40% are Hepatitis A, 40% are Hepatitis B and the remaining 20% are NANB. A subpopulation of HBV infected people are infected with a second virus called Delta hepatitis (4). It is a defective virus containing small circular RNA and depends on HBV for its infectivity. After infection, it dramatically increases the severity of the symptoms of HBV infection. There has recently been a growing interest in Delta virus as it seems that this virus has many similarities with some plant RNA viruses called viroids (5,6).

The frequency of infection by HBV varies very widely around the world. This variation becomes clear when one compares the frequency of chronic carrier in different parts of the world. For example, in developed nations like Europe and North America the frequency is 0.1 to 0.2% of the population, whereas in developing countries, in Africa, Asia and the Middle East, the frequency is 5% to 30% (7). In regions where the carrier frequency is low, most of the infections occur in dialysis patients, sexually promiscuous people, intravenous drug users, blood transfused patients and medical staffs. In contrast, in high carrier-prevalence

regions, a majority of infections occur in childhood and are derived largely from mothers, other carriers in the family or other infected children. Around 75% of the new HBV infections take place in the first 3 years of life (8). Because of this difference in the epidemiology of HBV infection in different parts of the world, the immunization strategy should also be different. In the high carrier frequency regions, mass immunizations are recommended, whereas in the low carrier prevalence regions, only the people in the high risk group may be vaccinated. (2).

The onset of symptoms after HBV infection is rather slow with only around 50% of the patients showing some kind of symptoms, the rest may have no signs of liver disease. In a typical infection, at one to four weeks, the person will feel rundown, showing symptoms of fatigue, loss of appetite, etc. (4). Since the different types of hepatitis share too many similar clinical symptoms, it is very hard to diagnose HBV infection without the help of a diagnostic tool. These diagnostic kits are based on enzyme linked immuno assay and use antibody against HBV. These are also routinely used by blood banks in the United States to screen all donated blood to stop the transfusion mediated transmission of the disease from chronically infected person. Although the parental route is the major route of transmission, it is now clear that HBV can also be transmitted by close or intimate contact. HBV surface antigen can be readily detected in the blood of the patient and the level rises with time. In the case of acute infection, with clinical improvement of the patient, the surface antigen titer falls and finally disappears. This is followed by the appearance of the antibody against surface antigen in the blood. This antibody stays in the blood for a long time and gives the patient immunity from further HBV infection. In the case of chronic or persistent infection, the titer of surface antigen stays at high levels for many years and the patient fails to show any antibody against it in his blood. A significant fraction of these carriers eventually die from hepatocellular carcinoma or liver cancer. It is interesting to note that more men are affected by liver cancer than women (9). One cannot rule out the possibility of the influence of different kinds of hormones in the body on the manifestations of these cancers. In support of this hypothesis, it has been found that HBV gene expression increases in some hepatocellular carcinoma cell lines when these cells are treated with corticosteroid hormones (10-12). Additional support came from the work of Shafritz and co-workers, who have identified a sequence in the HBV genome which responds to glucocorticoid and thereby helps to increase the transcription after hormonal challenge (13).

VIRUS

HBV, a member of a family of viruses called hepadna viruses, is the smallest known DNA virus among all the mammalian viruses. The other members in this family infect duck (14), woodchuck (15), ground squirrels (16) and tree squirrels (17). Although these viruses have considerable homology in their genome and the number of genes whose relative arrangement are very similar, they have very limited host range, i.e., each virus is highly specific to its host and does not infect others. The virus was believed to be liver specific, but in some studies, HBV has been found in bile duct epithelium, endothelial cells, smooth muscle cells of blood vessel walls and lymphoblastoid cells from bone marrow (18, 19). HBV particles can be detected in the serum of infected people. These are called "Dane particles" and have a diameter of 42nm (20). The HBV consists of a central core region containing a core antigen (HBcAg), DNA genome with a protein attached to it and an enzyme (reverse transcriptase) required to help the DNA synthesis (21). Surrounding the core region, there is an outer coat made of surface antigen (HBsAg). This surface antigen itself can form particles without any inner core. Such particles can also be found in infected blood and appear as 22nm spheres or as tubular filament of 22nm diameter with variable length. These two kinds of particles far out number the infectious virus particles in patient's blood. For example, in patient blood, the total number of particles could be around 10^{13} per m*l* whereas the number of infectious (Dane) particles would be around 10^{10} per

m*l* of the Serum (22). The function of these non-infectious particles is not clear. It is possible that the virus uses them as decoy antigen to escape from the immune system of the body.

Immunological studies have shown that there are some antigenic variations in HBV. Based on that, HBV has been subtyped in different groups as adr, adw ayr and ayw. There are some minor variations in the sequences of the genome of viruses of different subtype (23-28). The clinical importance of these differences is not very clear. It is believed that antibody against one subtype can neutralize the other types and hence one kind of vaccine may be effective against all the subtypes.

The genome of HBV is a partially double stranded circular DNA. The minus strand is the longer one and is a complete circle with its end not covalently closed. The plus strand is the incomplete one and the length of this strand is variable. The virus carries an enzyme which can elongate the plus strand after infection or *in vitro* when deoxynucleotides are added. The genome length is around 3200 bases and has many open reading frames. Interestingly, all the proteins are translated from mRNAs which are transcribed from one strand, the plus strand (21). A protein is covalently attached to the 5′ end of the minus strand (29, 30). The two strands are held together to form a circle by a 224bp overlap at the 5′ of plus strand. This overlap is flanked by two 10-12 bp direct repeats (DR1 and DR2) which play a central role in the replication of the virus (31-33). It is believed that with the help of these direct repeats the virus can make mRNA which is larger (3.8kb) than the genome size. It is this terminally redundant RNA from which new DNA is made by reverse transcriptase for replication. Thus, even though HBV is a DNA virus, one of its intermediates for DNA replication is RNA and in that respect, it has similarities with retro viruses. Apart from the 3.8kb RNA which also functions as the template for protein translation, there is another major RNA of smaller size (2.1kb). Both the RNAs terminate at the same site but have different initiation sites with the shorter RNA having multiple initiation sites. Both the RNAs have polyA tail at 3′ end. Both of these RNA can serve as messenger RNA and together they code for core antigen (C), surface antigen (S), polymerase (P) and protein X (21). A fifth antigen called e antigen has been detected in the serum of infected individuals. It has been shown that e antigen is generated from proteolytic cleavage of C antigen (34, 35). Recent evidence indicates that this cleavage could be mediated by C antigen itself (36). It is interesting to note that almost all the genome is used to code for proteins and so far no intron has been identified. A large region of the viral DNA is read in two different reading frames, giving rise to different proteins. This reflects how the virus has evolved to use the genomic information in a very efficient way. There are three translational initiation sites for the surface antigen gene. Due to the heterogeneity at the 5′ of the small RNA, only a fraction of them contain the second initiation site (37, 38). None of the small RNA contain the first initiation site. The most abundant surface antigen, however, is translated from the 3rd initiation codon and is called S antigen. The region between the first and the third initiation codons is called pre-S region. The S antigen can be in either of the forms-glycosylated and non-glycosylated, whose molecular weights are 24kd and 27kd and the proteins are called P24 and GP27 (39). The polypeptides initiated at the first and the second translation iniation sites comprise the minor forms of the surface molecules. They all are glycosylated with molecular weights around 33kd, 36kd, 39kd and 42kd. The proteins GP33 and GP36, also called pre-S2 protein, have the same run of amino acids and the difference in their molecular weights reflects the difference in the amount of glycosylation they have.

This also true for GP39 and GP42 which are called pre-S1 proteins. Pre-S1 and pre-S2 proteins are made of the 226 amino acids encoded by the S region plus an extra 163 (for pre-S1) or 55 (for pre-S2) residues at the amino terminal end. These extra amino acids are derived from the pre-S

region (39-41). Since pre-S containing antigen is a minor surface antigen in intact viron, early attempts to make a vaccine against HBV have ignored any significance of pre-S region. The major goal of those approaches was to find a very effective way to produce large quantities of S-antigen. Recent studies, however, indicate that the pre-S is the most immunogenic region in the surface antigen (42, 43) and is responsible for for binding to human polymerized albumin (44, 45). This region is also thought to be key element in attaching to the liver cell (46, 47). Other studies have shown the importance of pre-S in the assembly and secretion of surface antigen particles (48, 49). In light of these studies, it is generally agreed that in order to make a vaccine very potent, it is most reasonable to include pre-S region in the preparation.

VACCINE

It has been nearly thirteen years since small pox (Variola Major), one of the deadliest diseases, has finally been wiped off from the face of the Earth. The battle was not easy; it took international organizations, local teams and many field workers to finally snap the chain of infection in 1975. The key element in this paramount success was the availability of an effective vaccine against small pox and an exceptional cooperation at the international level. Similarly, in the fight against Hepatitis B, availability of a safe, cheap and effective vaccine is essential for us to eradicate the disease. Several approaches have been taken to make a vaccine against HBV. One of the obvious ways to produce vaccine would be to grow the virus in large amounts and use the inactivated virus as vaccine. Since the Hepatitis B virus has a very strict species specificity, the human virus cannot be grown in mouse or rabbit; the only animals which can be infected with HBV are non-human primates. Since the number of them available for experiments are very low, we cannot use them to grow large amounts of virus. Also, until recently (50, 51), all of the attempts to grow the virus persistenly in laboratory tissue culture have failed (52, 53). In order to curcumvent this problem, particles from the serum of chronic carrier of HBV have been isolated and purified. Purified preparation of such particles, which consist of both liver virus and the empty shell of HBsAg, are then inactivated and processed to make vaccine (54-56). Vaccines made from serum of human carrier are called serum vaccines. There are variations in the published procedures for purification and processing of the serum particles. In some cases the particles have been heavily degraded by protease treatment which results in the considerable loss of immunogenicity of the product. We know now that during such strong treatment the pre-S portion, which is the most immunogenic region of the surface antigen, is destroyed. Recently, other procedures have been developed where this region can be preserved during the inactivation process, so that only a fraction of the previous dose is necessary to elicit the same amount of immune response (57). Under electron microscope, the particle nature of the purified and processed vaccine can be observed. All the serum vaccines so far tested and used have been proved to be safe and they give protection against infection by all HBV subtypes (54, 55, 58, 59). When given to babies very shortly after birth, these vaccines can also reduce the perinatal transmissions, which is the major course of transmission in some parts of the world (60, 61). Several pharmaceutical companies are marketing this kind of Hepatitis B vaccine. Even though the price of such vaccines were initially very high, due to competition and better technology, these vaccines are becoming more affordable.

Human serum is not the only source of Hepatitis surface antigen. There are a few hepatic cell lines which have been established from the liver cells of chronic carrier. In most of these cases the hepatitis DNA is integrated into the host cell (63). For example, the PLC/PRF/5 cell line contained several copies of HBV genome in the integrated form (64-67). This cell line produces HBsAg but no other viral protein (68). The antigen which is secreted as particles of size 22 nm and 35 nm, contain no HBV DNA. Since there is no live virus present in the secreted product, the surface antigen

can be harvested and purified from the culture medium in the laboratory and, in principle, such preparation can be used as vaccine. However, when tested, it was found that the immunogenicity of such antigen was much lower than the vaccine derived from serum (69). Also, the amount of surface antigen produced by such cell line is not enough to make a vaccine with economic feasibility.

Even though serum derived vaccine has definite economical advantaged over the vaccine derived from hepatoma cell lines, it has some disadvantages of its own. Since the starting material is derived from the serum from patients who may have a number of other either known or unknown diseases, there is always some risk associated with introduction of such vaccine into the body. From the present body of data, it is clear that this kind of vaccine is very safe and nobody has gotten hepatitis from it, but the possibility still remains that such vaccine may carry agents for diseases which may manifest after a very long time. This kind of possible scenario and the recent epidemic of AIDS have made a substantial fraction of the population very reluctant to take this vaccine. The other major future problem will be when, with the help of vaccine, the disease will be under control and only few or none of the carriers will be around. This will cause a shortage in supply of infected serum. These considerations have prompted several workers to find out a way to generate substantial amounts of HBsAg in the laboratory, so that the product will be free of human serum and one can produce as much as necessary. Different laboratories used the techniques of recombinant DNA to achieve such a goal In general, the HBV DNA, either whole or only part of it containing the gene for HBsAg, was cloned in expression vectors and were expressed in heterologous systems. The surface antigen gene has successfully been expressed in E. Coli (27, 70), yeast (71-75), mouse (76-82), monkey (82-27), Chinese hamster (88-90) and human (90, 91) cell lines. Expression vectors derived from Bovine Papilloma (77-80), SV40 (82, 85, 86, 88-90) Adeno (87, 92) and vaccinia (84) viruses have been used for such purposes. In some cases, the recombinant constructs were introduced into the cells by cotransfection with other DNA with selectable marker like herpes simplex virus thymidine kinase gene (82). In other cases, selectable marker like dehydrofolate reductase (DHFR), which confers resistance to methotrexate to the recipient cells was incorporated in the plasmids (89, 90). The recipient cells were then challenged with increasing amount of methotrexate. This later procedure not only selects the cells harboring the plasmid DNA but also helps to increase the number of copies of the plasmid per cell. In principle, this would increase the gene dose and hence the level of transcription and expression of the surface antigen gene. However, when different clones were studied after methotrexate challenges, it was found that increasing gene doses did not necessarily contribute to higher expression level (89). Such lack of strict correlation was also found by other groups (80; author's own observations). This perhaps was due to the possibility that the complete functional surface antigen gene was not amplified equally in all clones. Expression may also depend on the chromosomal location of the integration of the foreign gene. One of the common observations made by all the groups was that the heterologous cells not only expressed the surface antigen gene but also assembled the polypeptides into particles of 22 nm diameter which had the same appearance as the particles isolated from human plasma. These particles have a boyant density of 1.2gm/m*l* and are devoid of any DNA in them. When produced either in *E. Coli* or in yeast, the antigen could not cross the wall and the cells them. When produced either in *E. Coli* or in yeast, the antigen could not cross the wall and the cells had to be broken up to harvest the antigen. Using mammalian cells have unique advantages in this area; they secrete the antigen very efficiently into the tissue culture medium from where it can be harvested and purified. The mammalian cells also glycosylates the surface antigen where as the bacterial system cannot. Since it is important to have proper glycosylation when the antigen is used for vaccine, different laboratories are concentrating their effort in expressing this antigen in eukaryo-

tic system with maximum efficiency. With present technology, one can get around 15 ug of antigen/10^6 cells/day in such system (89). Such expression level is considered high enough for economic feasibility. Vaccine, derived from antigen expressed in yeast has been produced and found to be safe and effective. Such vaccines at present are commercially available and are more expensive than some serum derived vaccines. However with time and more competition, when the price will come down, the recombinant vaccine is expected to be very popular since it is not derived from human serum which may contain causative agents of infectious diseases.

Extensive research is going on in developing other kinds of hepatitis vaccine. One of these approaches is to identify the most important regions on the surface antigen and synthesize a small polypeptide covering that area. Such synthetic polypeptide, when injected into an individual, will make antibodies which in turn will recognize the intact virion. Results from different laboratories show promise in establishing such vaccine, although some of the antibodies produced have lower affinity compared to those made by natural antigen (93-96). Anti-idiotypic antibody is also being considered to be used as vaccine (97). These are antibodies against the antigen. Both, the antigen and the anti-idiotypic antibody, bind to the antibody against the antigen indicating that they carry similar epitopes. Thus, antibody against the anti-idiotypic antibody should bind the antigen through those shared epitopes and may neutralize the virus. In other words, injecting the anti-idiotypic antibody will confer immunogenicity against the virus (98). There are a couple of advantages in using anti-idiotypic vaccine over the conventional one. First of all, it is much cheaper to produce antibody than to produce antigen in tissue culture and secondly, the viral antigen, along with its major epitopes, may also carry some other epitopes which will be very similar to some of body's own protein (99). Antibody raised against such epitopes may trigger autoimmune disease and by using anti-idiotypic vaccine, one can reduce such risk.

With such extensive research, commercial interest and growing concern about Hepatitis, one can hope that within the next ten years the spread of the disease can be brought under total control. Recently, one of the biotechnology companies has agreed to make a serum derived Hepatitis vaccine available to an international organization at a very inexpensive price for mass immunization (62). With this kind of international cooperation, care and understanding, complemented by advancement in knowledge and technology, the human race will soon win the final battle against Hepatitis B.

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