2.1 Overview of Hepatitis

Hepatitis is an inflammation and infection of liver tissue, mostly caused by virus. Other causes include use of heavy alcohol, certain medications, toxins, autoimmune diseases [Beck and Nassal, 2007]. It may be a temporary or long term infection that depends upon the duration of infection *i.e.* less than or more than six months [Chen *et al.*, 2015]. Viral Hepatitis are essentially five types viz. A, B, C, D and E. Various viruses are responsible for such of different types of Hepatitis.

Hepatitis A is caused by Hepatitis A virus (HAV) and gets easily transmitted through contaminated food and water.

Hepatitis B is caused by Hepatitis B virus (HBV) and gets transmitted through infectious body fluids viz. blood, semen or vaginal secretions, sex with infected partner *etc.* It was reported that 350 million people suffer from Hepatitis B worldwide.

Hepatitis C is caused by Hepatitis C virus (HCV) and gets transmitted through direct contact with infectious body fluids, sexual contact and injection. Approximately 2.7 to 3.9 million Americans suffer from Hepatitis C.

Hepatitis D is also called as delta Hepatitis. It is caused by Hepatitis D virus (HDV) and gets transmitted through direct contact with infected blood. Hepatitis D is a very rare type occurs only in conjugation with Hepatitis. It multiplies only in the presence of Hepatitis B infection.

Hepatitis E is a waterborne infection caused by Hepatitis E virus (HEV) and found prevalent in poor sanitation area.

2.2 Geographical epidemiology/distribution

Hepatitis B virus infection is found high prevalent in sub-Saharan Africa and East Asia regions, where adults are chronically infected at 5–10%. Amazon and southern parts of eastern and central Europe are also fall under high incidence area. Middle East and India are accounted for estimated 2–5% persons with chronically infection.

- Approximately, 1 in 12 persons worldwide are living with chronic viral Hepatitis B.
- One third of world's populations carry this infection.
- An estimated 350 million people are living with chronic hepatitis B worldwide.
- 15-25% of people die due to liver related diseases
- Reported cases of 1 million deaths annually

Figure 2.1: Worldwide prevalence of Hepatitis B carriers and primary hepatocellular carcinoma (Courtesy Centers for Disease Control and Prevention, Atlanta)

2.3 Hepatitis B virus

HBV is a noncytopathic virus where virus itself does not cause direct damage to liver cells. Instead, the aggressive immune response to the virus causes inflammation and damage to the liver.

- Hepatitis B virus is a double-stranded DNA virus belonging to the Hepadnaviridae family of viruses (Figure 2.2).
- All viruses belonging to the same family infect other vertebrates, but Hepatitis B virus infects only humans and chimpanzees.
- Genome is found circular in double-stranded DNA viruses (app. 3.2 kb in size) which replicate, unusually, by reverse transcription.
- HBV has compact unique body, which has four overlapping reading frame running in one direction and no any noncoding regions.
- These four overlapping open reading frames (ORFs) in the virus genome are responsible for the transcription and expression of seven different Hepatitis B protein substances [Sommer *et al.*, 1997].
- HBV genome also participates in regulation of transcription, polyadenylation site determination and encapsidation into nucleocapside [Sommer *et al.*, 1997].
- The Hepatitis B virus consist an outer lipid envelope. An icosahedral nucleocapsid core is also found which is composed of protein molecules [Alexander and Volker B, 2009].
- The HBV genome has special S gene, which code for HBsAg. S gene region consists of two pre-S region. These two pre-S region genes coded the binding sites of hepatocyte receptors (Figure 2.3).

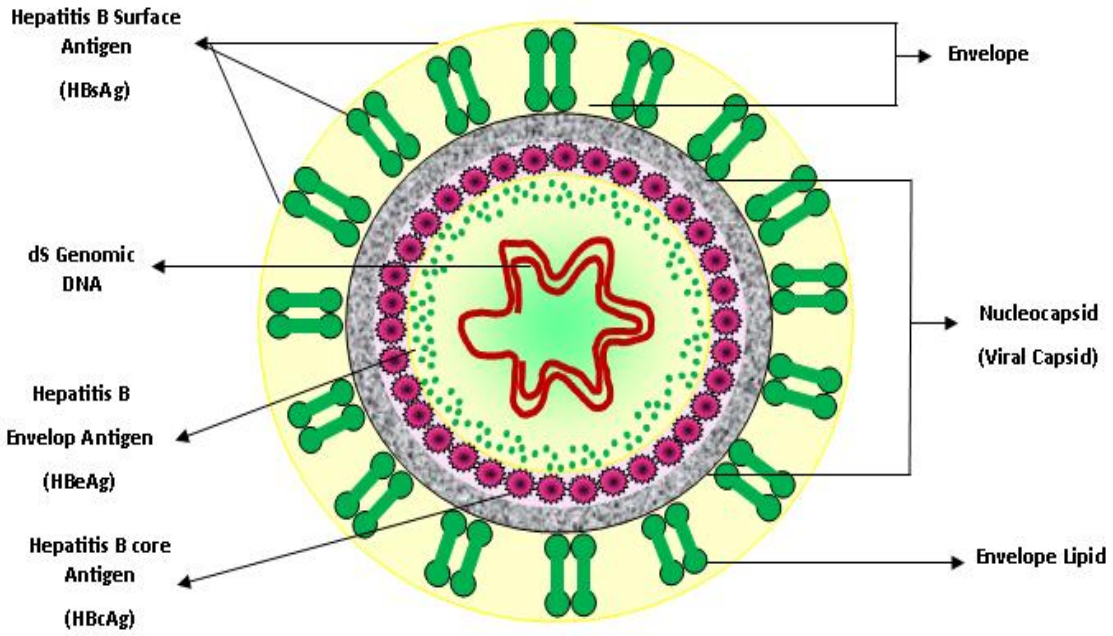

Figure 2.2: Complete assembly of Hepatitis B virus or virion, containing Hepatitis B surface antigen (HBsAg), Hepatitis B envelope antigen (HBeAg), Hepatitis B core antigen (HBcAg) and double standard genomic DNA

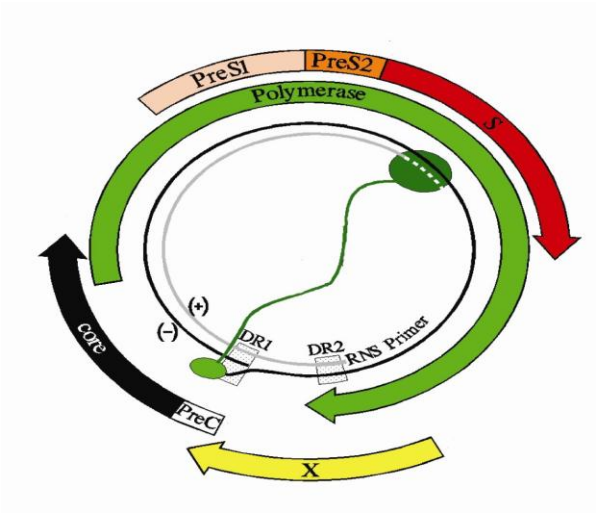

Figure 2.3: Genome DNA sequence frame of Hepatitis B virus. The four encoded sequences are C-core, S-surface proteins, P-polymerase, and X protein. The sequence C-core and P-polymerase presents in same orientation and open. While the S-surface protein translational frame overlaps these reading frames [Liang TJ *et al.*, 2010]

Another is P gene, which codes the DNA polymerase. Apart from P and S gene, another C gene is present in the genome which codes the HBcAg and HBeAg. X gene is that also found that activates viral as well as cellular promoters [Akarca and Lok, 1995; Naoumov *et al.,* 1992].

2.4 Hepatitis B virus replication

HBV replication followed well defined stages like binding to the surface of host cells and entry of virus inside the cells. The virus enters in the cell cytosol, then start to uncoat the nucleocapsid and initiate cccDNA followed with transcription and translation of virus-specific genes. Later prepared a complete assembly of capsids and start reverse to transcription process. This whole process is regulated by both host and viral factors [Chen *et al.,* 2015]. Viral replication involves three phases:

High replicative phase:
- Detection of HBsAg, HBeAg, and HBV DNA in blood serum
- Increased levels of aminotransferases
- Histologic changes in liver
- Inflammation

Low replicative phase:
- Low levels of HBeAg and HBV DNA
- Appearance of anti-HBe
- Histological changes
- Decrease in inflammation

- Seroconversion (loss of HBV DNA and HBeAg)

Non replicative phase:

- Not easy to detect in serum
- Require highly sensitive technique for detection
- Reduction in inflammation
- Improvement in histologically
- Development of cirrhosis

2.4.1 Viral infection and host cell attachment

The mechanism of attachment of Hepatitis B virus to the hepatocyte cells is not completely understood but it was assumed that, it could be due to the binding of virus to surface receptors of hepatocytes cells. The eukaryotic or host cells have group of cellular protein known as annexins. There by virus easily gets attached to this protein. The annexin is group of calcium containing protein, which is indirectly related to the cell membrane phospholipids [Klingmuller and Schaller, 1993]. The virus nucleocapsid contains HBV-DNA and gets transported into the nucleus, where replications occur. Later, the single stranded DNA converts to form covalently closed circular DNA (cccDNA) near the nucleus. Then the host cell transcribes DNA into RNA with help of RNA polymerase. Few of the transcribed RNA enter the cytoplasm and some compose with HBV polymerase for encapsidation by HBcAg. Then, get converted to mature double stranded DNA (dsDNA) inside the capsid by reverse transcription. The developed new virion is accomplished by process of budding that involves HBcAg through endoplasmic reticulum membrane. After the completion of this process, virion

starts to separate the cells and leads to exocytosis (Figure 2.4) [Francois *et al.*, 2001; Glebe and Urban, 2007].

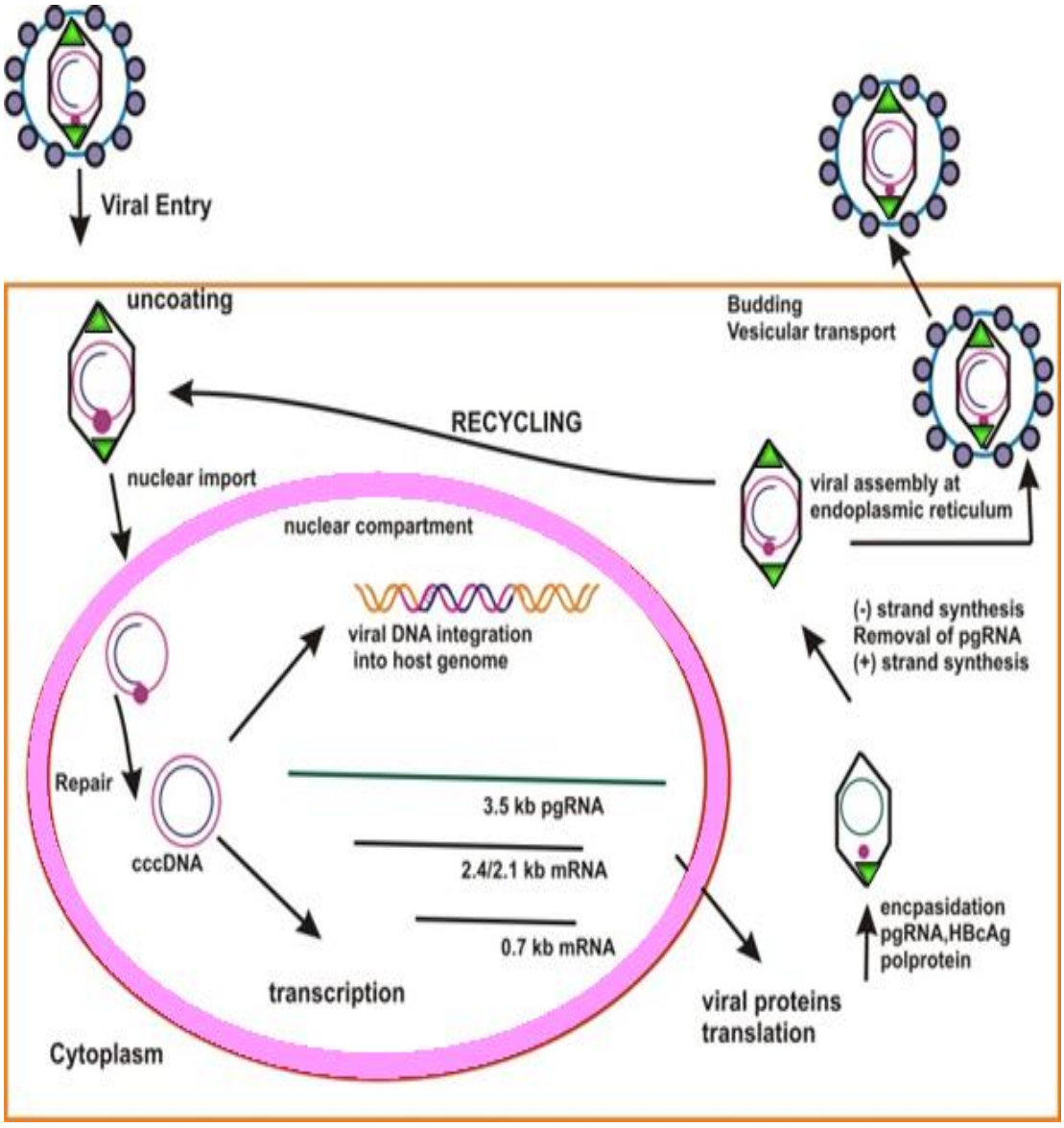

Figure 2.4: Life cycle of Hepatitis B virus. Attachment of virus on surface of host cells, replication and finally release of viral like particles which further develop to form completed assembly

The blood serum of infectious person showed three different viruses like particles observed by electron microscopy. The two viral particles are at 20 nm diameters with smaller spherical structures. Their filaments are variable in lengths but fixed width at 22 nm. These spheres and filaments are comprised of HBsAg, but no viral nucleic acids and host-generated lipids. Therefore, it is non-infectious [Gavilanes and Gonzales-Ros, 1982; Liang *et al.*, 2009].

However, HBV infectious virion has a smaller particle that contains a double standard genomic structure surrounded by lipid envelope. The inner most layer is nucleocapsid that contains complex of HBcAg [Gerlich and Robinson, 1980]. The following are the three major types of Hepatitis B antigens that encodes for the genomes of HBV:

(1) Hepatitis B Surface Antigen (HBsAg): Hepatitis B virus release a smaller particle of 22 µm diameter called as HBsAg. It is hydrophobic and contains a highly antigenic epitopes, which may be responsible for the prompting of immune response [Yen *et al.*, 2002]. When the HBsAg is detected in person blood sample, it is obvious that the virus is still alive in the liver and has a chance of transmitting to another person. If both negative HBsAg and positive anti-HBe are present in the blood sample, it is means the virus is inactive. But when a person carries HBV infection for several years, it means that he/she is suffering from chronic Hepatitis B. There is chance to have "precore mutant" of HBV because of the presence of both negative HBsAg and positive anti-HBs [Saikia *et al.*, 2007].

(2) Hepatitis B core Antigen (HBcAg): This type of antigen cannot be directly detected by blood test. The antigens are need to be isolated and analysed using an infected hepatocyte [James and Ellen, 2002].

(3) Hepatitis B early Antigen (HBeAg): Presence of early antigen indicates high level replication of the virus in the sample. This type of HBV can be noticed during an acute HBV infection. This antigen can be easily detected through normal blood test [James and Ellen, 2002].

2.4.2 Genomic replication of Hepatitis B virus

In Hepatitis B viruses the double standard genome DNS replicate in three phases:

(1) The viral genome enters the icosahedral core. The viral genome is 3.2 kb length, circular and has partially double-stranded DNA. This partially double-stranded DNA does not covalently bind to each other therefore, it is easy to separate and replicate.

(2) On binding of Hepatitis B virus on surface of host cells, the host cells generate the plasmid like structure circular DNA (cccDNA).

(3) Circular DNA (cccDNA) produces genomic RNAs by transcription with the help of RNA polymerase II. These RNAs are packed inside the capsids, where they RNAs convert to DNA genomes by reverse transcription.

Immature RNA does not contain nucleocapsids but matured rcDNA (relaxed circular DNA) contains nucleocapsids, which further involved in amplification of intracellular cccDNA and gets released from the enveloped progeny virions [Beck and Nassal, 2007].

2.5 Hepatitis B virus Transmission

Replication of Hepatitis B virus occurs highly in blood (108 to 1010 virions/mL), especially during acute condition. Virus potentially spread out by parenteral or mucosal exposure of infected blood [Gerberding *et al.*, 1994]. Virus can also be communicated between family members within households, possibility through contact of mucous membrane or non-intact skin or saliva containing HBV and body fluids such as seminal, vaginal or menstrual fluid and saliva fluids [Gentile and Borgia, 2014]. Incubation period of the disease differ from 30 to 180 days. There are mainly two types of mode of transmission of Hepatitis B virus:

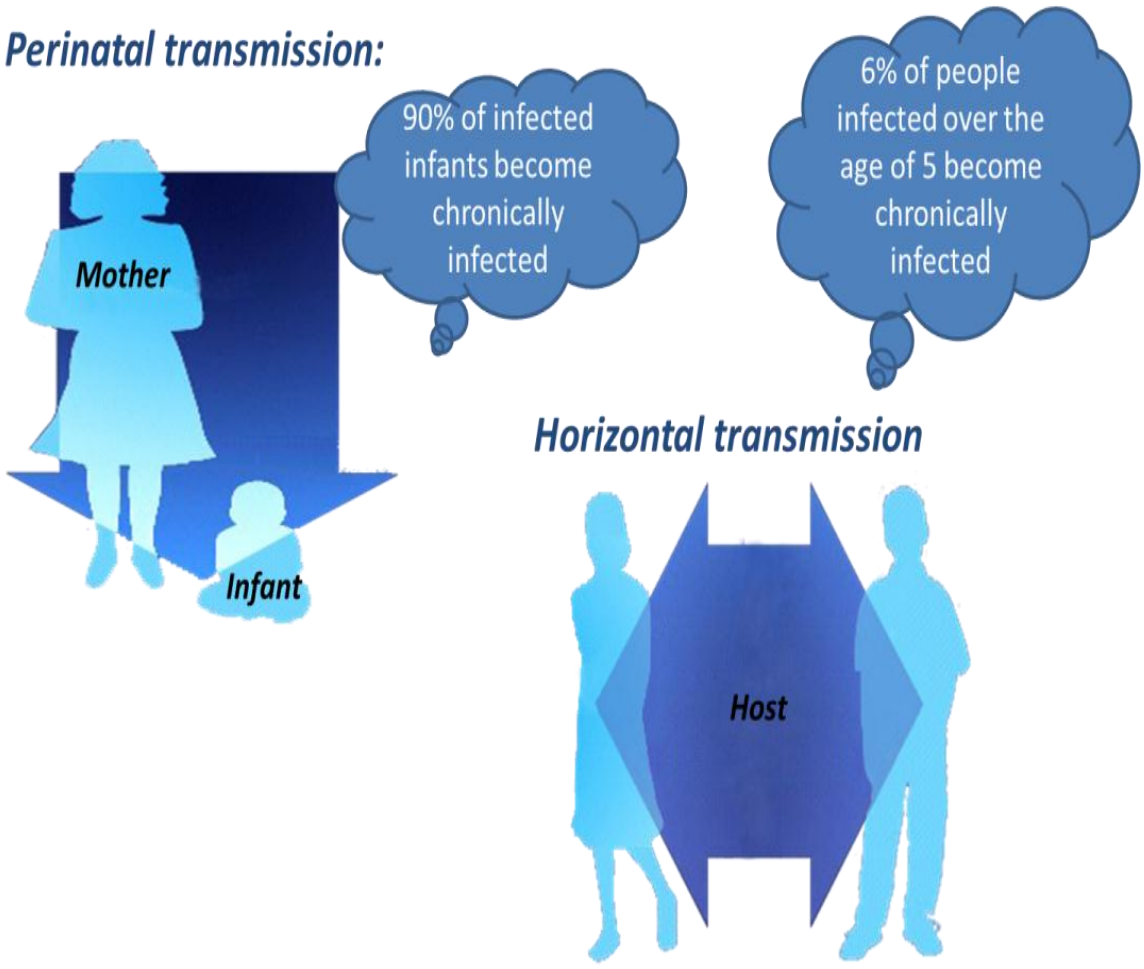

Figure 2.5: Hepatitis B virus transmission: (1) Perinatal transmission and (2) Horizontal transmission [Lee *et al.*, 2006]

(1) Perinatal/vertical transmission: 90% of the infected infants become chronically infected by this type of perinatal transmission. For example: The vertical transmission of viral from mother to child (MTCT) during child birth. Proper immunoprophylaxis arrest the transmission of HBV in child through breast feeding [Shi *et al.,* 1994].

(2) Horizontal transmission*:* 6% of people with age >5 become chronically infected by horizontal transmission [Hughes, 2002; Biddeberg *et al.,* 2008; Fairley and Read, 2012]. The different possibility of transmission of HBV includes:

- Sexual transmission from unvaccinated men who have sex with multiple partners or contact with sex workers.
- Transfusion of blood or other blood products.
- Infection occurs during medical, surgical and dental procedures.
- Contaminated needles and syringes reuse.
- Body fluid of HBV infected patient plays substantial role in horizontal transmission.

The main major routes of transmission of Hepatitis B virus are intravenous injection of drugs, tattoos, sexual or maternal/infant transmission at birth *etc*. When mother is noticed with positive HBsAg and HBeAg at the birth of baby's, it is very necessary to understand the mechanisms of HBV transmission during pregnancy. These mechanisms can be targeted to prevent the transmission. The maternal to infant transmission of HBV can occur *via* three routes: intrauterine transmission; transmission during delivery; and postpartum transmission [Gitlin, 1997].

Minor cases of intrauterine transmission are observed in case of HBV transmission. It can occur in two ways, first trough the transfer of HBV occurs of placental barrier to

reach fetus and second during its passage [Zhang *et al.,* 2004; Bai *et al.,* 2007]. Transmission of HBV during delivery is the most common method of vertical transmission of virus. It is mostly due to contact of new born with mother. The infected secretions or blood of the mother at the time of delivery of baby's. [Piratvisuth *et al.,* 2013]. During and after child birth, both mother and baby may acquire infection due to very close contact [Degli and Shah, 2011].

2.6 Clinical aspects of Hepatitis B virus

Hepatitis B virus can cause both acute and chronic type infection. The symptoms of acute and chronic infections generally vary and also depends upon the incubation period. When infection is more than six months, the clinical sign chances to chronic infection. The first instance of virus attack, it called acute infection or initial phase of infection (or a new infection). First time infection of healthy person does not show any symptoms of Hepatitis B hence it is difficult to recognize the disease. It can be characterized only through occurrence of HBsAg and HBcAg in blood. Most of the cases where acute liver failure occur in patient. The acute infection can be easily cured and does not require any liver transplantation. It can be prevented through development of strong and high immune response to against the entry of virus. This sub-clinical acute infection has greater risk of conversing into chronic carriers [Krugman *et al.,* 1979; Hoofnagle and Di Bisceglie, 1991].

2.6.1 Hepatitis B acute/chronic

The incubation period of acute Hepatitis or the period from exposure to development of jaundice is 60–150 days or about 90 days. In case of acute Hepatitis, the development of symptoms of infection is age-dependent. Mostly 90% perinatal Hepatitis B virus

infections are asymptomatic. Although 5–15% are typical manifestations of acute hepatitis, it is generally observed in newly infected young children (1–5 years of age) and in 33–50% of older children, adolescents, and adults [McMahon *et al.,* 1985]. Nausea, vomiting, dark urine, fever, jaundice, changes in stool colour, and splenomegaly or hepatomegaly are common signs and symptoms of acute Hepatitis B. The first detectable serologic markers of acute HBV infection are Hepatitis B surface antigen (HBsAg) and antibodies to HBcAg. But after the infection of 6–12 months, immunoglobulin M antibody developed against Hepatitis B core antigen becomes undetectable and the Hepatitis B core antigen persists for life to chronic infection. On recovery of person from HBV infection, the HBsAg and developed antibody gets completely eliminated from the blood. Development of anti-HBs antibody indicates immunity against HBV infection. Various serological markers of Hepatitis B at acute and chronic stages, post and during infection, and after vaccination are represent in Table 2.1 [Seeff *et al.,* 1987; Ortiz-Interian *et al.,* 1990; Davis *et al.,* 1995].

A normal blood test is sufficient for diagnosis of Hepatitis B, It may be either acute or chronic condition. If people failed to identify/diagnose the infection within six month, it starts to chronic stage. Chronic hepatitis B virus infection is mainly divided into four different phases.

I. **Immune tolerance phase:** In this phase, viral replication is highest but the damage of liver is minimum or very low. The level of alanine aminotransferase (ALT) is normal.

II. **Immune activation phase:** In this phase, the immune system attempts to clear the virus from body. Replication of virus decreases, level of alanine

aminotransferase and inflammation increases. Hence, histology of liver biopsies is required.

III. **Immune surveillance phase:** In this phase, DNA of Hepatitis B virus is low and not detectible in blood. The level of alanine aminotransferase is normal. The histology of liver is normal or improved due to conversion of HBeAg to anti-HBe.

IV. **Reactivation phase:** This is the last stage during which amount of virus increases and the level of alanine aminotransferase also increase.

The symptoms cannot be easily identified in year or months. However, the most general symptoms include:

- Abdominal dysfunction
- Extreme Joint pain
- Dark colour of urine
- Loss of appetite
- Yellow colouration of eyes sclera
- Fever
- Weakness

In case any such symptoms were observed, immediate treatment is required. Consult the doctor, if you have been exposed to virus infection.

2.6.2 Serologic and biochemical features

The presence of serological marker in blood serum sample indicates the disease condition, *i.e.* either acute or chronic condition. The first and foremost serologic marker of Hepatitis B virus infection is HBsAg, which is detected from 2-12 weeks, after the virus infection. The hepatic abnormality is observed with biochemical change and that can detected in 6-8 weeks of infection. The detection of Hepatitis B core antigen (HBcAg) against IgM antibody and detection of HBsAg usually takes place after 2 weeks of infection. After six months progression of infection, another core antigen antibody (anti-HBc IgG) is detected and identified. Small amount of HBeAg is noticeable in acute HBV infection in serum. The presence of HBeAg in serum shows highest infection of virus. Quantification of the Hepatitis B virus involves various pathology techniques.

Table 2.1: Hepatitis B markers and its probability in various infection conditions

Markers	Acute infection condition	Chronic infection condition	Post infection condition
HBsAg	+	+	-
Anti-HBs	-	-	+
HBeAg	+ Early, then -	±	-
Anti-HBe	- Early, then +	±	+
Anti-HBc- IgG	+	+	+
Anti-HBc- IgM	+	-	-
HBV-DNA	+ Early, then -	±	-
ALT	Increased (noticeable)	Increased (mild-moderate)	Normal

Quantification of HBV DNA assay is important in determination of on-going viral replication in patient blood serum. The detectable markers observed after the Hepatitis B virus infections are shown in Table 2.1. During acute conditions, aminotransferases especially alanine aminotransferase (ALT) increases 3 to 10 times. Generally the concentrations of ALT are higher than the concentrations of aspartate aminotransferase (AST). In case of liver failure, sudden fall of ALT and AST levels can be noticed [Gitlin, 1997].

2.7 Immune pathogenesis of HBV infection

When virus enters the body firstly it reaches the liver and infects hepatocytes and viral replication is mostly found in Kupffer cells of the hepatocytes [Hosel *et al.*, 2009]. After the infection the innate immunity becomes activated but the response is weak. Therefore, the clearance of virus and reduction of live infection completely depends upon the adaptive immune response [Chisari *et al.*, 2010]. It mainly involves CD8+ T cell response that are specific for HBV [Das and Maini, 2010]. Asabe *et al.*, (2009) also suggest that stimulation of immune response specific to CD8+ T cells are also depends upon CD4+ T cells. The CD8+ T cell is a class of effector cells that having capability of recognizing the pathogen and directly killing the infected cells or protein antigen or tumor tissue.

Whereas, inflammatory marker like interleukin-6 appears in response to viral attack followed by activation of regulatory expressers likes mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK). These inturn regulate the hepatocyte nuclear factor 4α (HNF4α) and HNF1α. They are the transcription factors of Hepatitis B virus expression [Quasdorff *et al.*, 2008]. Interferons also play an important role in regulation of MSC-I (major histocompatibility complex) class I antigens, present on cell

the surface. The key functions of MHC class I antigens is to identity and eliminate the infected cells. While, MHC class II immune response is restricted to capsid proteins (HBcAg and HBeAg) [Modrow *et al.,* 2003]. When virus enters the cell, they grow rapidly and replicate continuously. The infected cell, immediately activate the innate immune system and ready to rapid production of IFNα/β [Samuel, 1991]. Immune system is the combination of both adaptive and innate immune response. The generation and production of protective immunity system requires input from both the adaptive as well as innate immunity [Janeway *et al.,* 2005].

2.7.1 Adaptive and innate immune systems

Generally in the adaptive immune system antigen presenting cells (APCs) are involved. They are mainly B cells, various macrophages cells, and dendritic cells (DCs). Whereas, in case of antigen antibody mediated immune system, reaction B cells are involved, this type of antibody mediated immune reaction is also known as humoral immunity. Dendritic cells (DCs) and macrophages are responsible for the cellular immunity or otherwise called as T cell immune response. All the three antigens presenting cells (APCs) are responsible for the capture of pathogens, but in cellular immunity DCs are generally considered to be the most important in comparison than other two for the induction, generation and orchestration of adaptive immune responses [Steinman and Banchereau, 2007; Banchereau and Steinman, 1998]. Antigen presenting Dendritic cells (DCs) mostly exist in immature tissues and blood stream. When any type of pathogens enter the cells or tissues, the innate immune system wake up and produces various signals on release of DCs. The growth and maturation of dendritic cells occur at course of migration and maturation process in lymph nodes, whereas they present inside the native T cells [Jensen, 2007; Savina and Amigorena, 2007].

When any types of protein antigens or pathogens enter the cell, they will be typically captured by APCs by the process of phagocytosis or receptor-mediated endocytosis. Later protein antigens or pathogens degrad into peptide fragments in endosomal area of compartments. Depending upon type of cytokine signals generated, the histocompatibility complex (MHC) class II molecules to CD4+ T cells can be differentiated into a number of effector cells with either regulatory (Treg) or helper cells [Coffman, 2006; Dong, 2008]. In case of vaccine delivery system, the subtype of dendritic immunology CD4+ T cells viz. Th1 and Th2-type generate immune responses, which is generally very important for antigen antibody mediated immune responses and also important for the clearance of intracellular antigen and pathogen. In case of viruses, the cytosolic antigen comes into the intracellular part and gets degraded by APCs. Also gets fragmented into small protein like peptide fragments with help of MHC class I antigens to CD8+ T cells [Heath and Carbone, 2001; Rock and Shen, 2005; Vyas *et al.*, 2008].

When microbes come in contact with physical barriers like intestinal mucosa or skin, it is immediately recognized by Toll-like receptors, which further activate the immune cells [Takeda *et al.*, 2003; Barton and Kagan, 2009]. TLRs combined with Interleukin-1 receptors are known as the "interleukin-1 receptor/toll-like receptor superfamily". Immunostimulatory signals provided by TLR ligand binding cause the DCs to undergo prepare and secrete cytokines, and increase expression of MHC molecules as well as its co-stimulatory molecules [Iwasaki and Medzhitov, 2004]. In case of vaccine development, when the antigen presentation without any sufficient co-stimulation can cause T cell production [Schwartz, 2003; Mueller, 2010].

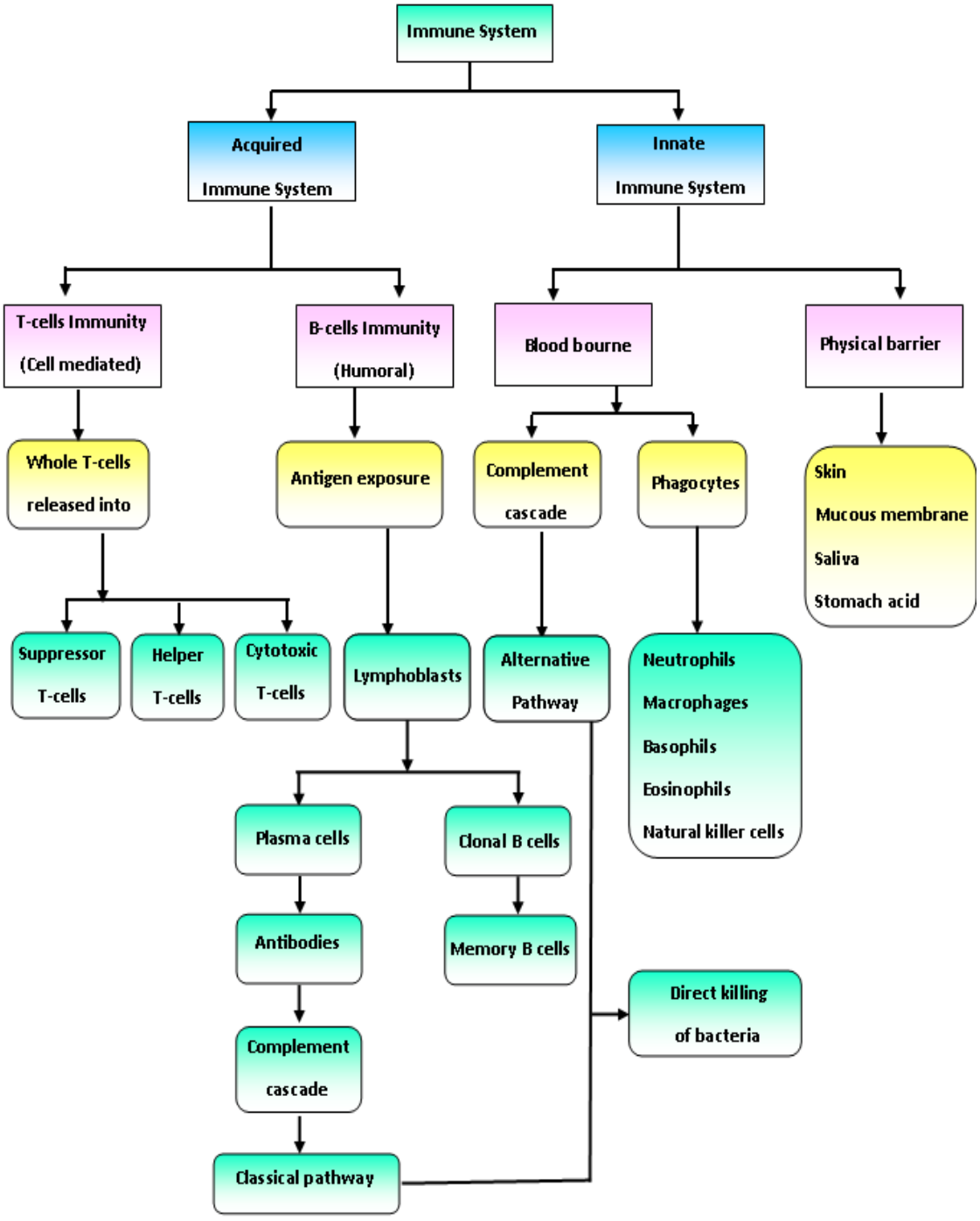

Figure 2.6: Schematic diagram of immune system representing acquired and innate immunity with involvement of different immune cells followed various pathway

2.7.1.1 The CD4 T cell response

In case of Hepatitis B infection, CD4 T cells plays a prominent role in elimination of virus in patient suffering from acute condition but in chronic condition, the activity of CD4+ T cells are less or weak response. Experimental result indicates that CD4+ T cells are not involved in clearance of viral cells and damage. Probably CD4+ T cells indirectly act on virus cells and control the infection by regulating of CD8+ T cell response and virus-specific B cells [Thimme *et al.,* 2003].

2.7.1.2 The CD8+ T cell response

CD8+ T cells play a central role in the prevention of external pathogenesis and clearance of virus. Many polyclonal CD8+ T cells are detected in peripheral blood of patient, suffering from acute Hepatitis B. But the polyclonal CD8+ T cells are weak during chronic infection patient blood sample [Penna *et al.,* 1991; Rehermann *et al.,* 1995; Bertoletti *et al.,* 1991]. Few studies examined the relationship between the virus specific CD8+ T cells and its effectiveness against infection. When the replication or growth of virus in patient blood increases the number of CD8+ T cells was also found more. While the replication of virus is independent of liver damage. HBV-specific CD8+ T cells plays a significant role in controlling Hepatitis B virus replication [Maini *et al.,* 2000]. The clearance of virus occurs due to CD8+ T cells, which depends on generation of specific IFNγ and IFNγ-inducible genes. Importantly, decrease in activity of CD8+ T cells is associated with decline in clearance of the virus. It is concluded that liver disease specific and viral clearances are mediated by specific CD8+ T cells [Thimme *et al.,* 2003].

2.8 Mechanisms of Hepatitis B virus clearance

It is well known and accepted that the CTL cells response are responsible for clearance of viral. The pathogenetic and noncytopathic anti-viral response in cytotoxic T cells action had been studied in Hepatitis B virus induced a transgenic mouse which develops an acute necro-inflammatory liver disease [Moriyama *et al.,* 1990; Ando *et al.,* 1994; Guidotti *et al.,* 1996]. In this developed model, cytotoxic T cells rapidly trigger two events, either hepatocytes apoptosis or secretion of interferon gama. This IFN-γ noncytopathically prevents the viral replication and inhibits the gene expression of rest hepatocyte. After that, viral nucleocapsids disappear in hepatocytes cytoplasm and also destabilized the viral RNA in nucleus, then the hepatocytes cells become perfectly healthy. Finally the transgenic mouse model showed that the CTLs related antiviral functions and cytopathic are completely independent and indicates a strong CTLs response, capable for clearance of Hepatitis B virus and suppression of viral genomic replication [Guidotti *et al.,* 1994; Guidotti *et al.,* 1996]. Viral replication is also supressed by involvement of many cells like natural killer T cells, CD4+ T cells and activation of toll-like receptor in transgenic mice model. It is also reported that Hepatitis B virus replication is suppressed by interferon alpha/beta due to antiviral effects [Cavanaugh *et al.,* 1998; Pasquetto *et al.,* 2000].

2.9 Diagnosis of Hepatitis B virus infection

The detection of Hepatitis B virus infection in serum is measured in presence of viral antigens and antibodies which is produced against the viral antigen. Routinely, the viral antigen is measured in patients suffering from Hepatitis B virus. Generally HBsAg is used as the key indicator of Hepatitis B carriers in both the acute as well as chronic condition.

The main markers of acute Hepatitis B infection are IgM antibody, HBcAg and anti-HBcIgM. The acute Hepatitis B infection persists for at least six months. The presence of virus in blood serum can be measured by detection of viral DNA by using polymerase chain reaction (PCR). Presence of HBsAg in blood serum for more than 6 months may give positive or negative results and called as chronic Hepatitis B carriers. After treatment, firstly the HBeAg disappear in blood, and observe anti-HBe. Similarly, HBsAg in serum is reduced and developed anti-HBs, due to presence to HBsAg [Xu *et al.*, 2002; Rock and Shen, 2005].

2.9.1 Treatment of Hepatitis B

Currently, no well-established treatment is available for acute Hepatitis B infection; it is only available for chronic condition. The treatment is based on induction of host mediated immunological control and reduces HBV-DNA levels in blood serum though conversion of HBeAg to anti-HBe. The main focus on treatment of Hepatitis B is reduction of any histological inflammation and control of disease progress. There are two treatment options [Lindh *et al.,* 2008]:

I. Interferon clears the virus from host's cells and enhances immune system.
II. Nucleoside/nucleotide analogues act as an anti-viral effect in host's cells.

2.9.2 Vaccines against viral Hepatitis B infections

The World Health Organisation recommends that "Hepatitis B infection can be effectively prevented by vaccination and should be included in childhood vaccination programmes in all countries." The History of Hepatitis B is as follows.

The first plasma-derived HBV vaccine: At the beginning of the 1980s, vaccination is available against hepatitis B infection [Lemon and Thomas, 1997]. In 1982, first time vaccine on the market is licensed by USA. It contained purified 22 nm HBs-Ag vaccine. It is virus inactive, plasma derived and no risk of transmission of Hepatitis B. Aluminium hydroxide was added which act as an adjuvant and thiomersal, it is preservative added to the vaccine. It has produced good protective antibody levels. But the production of antibody is limited, not sufficient as per the need [Payton *et al.*, 1993].

Recombinant vaccine: The first recombinant vaccine was developed in 1989, which was yeast species *Sackaromyces Cerevisiae* derived HBsAg recombinant vaccine. It was prepared by recombinant DNA technology, adsorbed on aluminium hydroxide using thiomersal as preservative. The main advantage of this vaccine is thermo stability, therefore easy to carry and distribute. The drawback of this vaccine is higher production cost and lower mean antibody titres [McAleer *et al.*, 1984].

New HBV vaccines: Various new traditional HBs-Ag-containing vaccine has been developed by the prompted use of pre-S sequences of virus.

Drug therapy: In case of acute Hepatitis B infection, medicine or treatment is not required, since patients spontaneously clear their infection. The universal symptoms of infection mainly include anorexia, vomiting, nausea *etc.* In chronic Hepatitis B viral infection, the symptom is very different from acute infection. Now a days, many medicinal agents are available for examination and diagnosis of chronic Hepatitis B. Mostly drugs are ineffective in normal dose and too toxic at effective doses. The goals of the therapy is to suppress the growth of virus, prevention of inflammation, reduction of liver disease and an enhanced long-term projection [Gitlin *et al.*, 1997].

Immunomodulators: These agents modify the function of immune system. This class of drugs either stimulate or supress the immune system. Immunomodulators mainly act on T cells and enhances its function also stimulates the production of interferons and interleukin levels [Lango and Lindblom, 1993].

Interferon: Interferon-α is one of the important interferon belongs to the naturally occurring proteins family, and act as both antiviral as well as immunomodulatory. They encourage the T-cell helper activity and inhibit T-cell suppressors. They also cause maturation of B lymphocytes and enhance HLA type 1 expression. The adverse reaction observed during therapy is shown in Table 2.2. Some of the drug causes dose dependent side effects due to continued therapy; some never resolve the termination of therapy [Wong *et al.*, 1993]. After the injection of interferon drugs, immediately showed side effect like fevers, myalgia, rigors, fatigue, arthralgia, and headaches.

Table 2.2: Interferon drugs and their adverse effects

Constitutional problems	Illness, arthralgia, fever, rigors, myalgia, fatigue
Hematologic condition	Thrombocytopenia, Leukopenia
Autoimmunity	Diabetes, hypothyroidism
Neuropsychiatric condition	Depression, irritable, insomnia

Currently available conventional vaccines are inefficient for generation of immune system, due to lack of suitable adjuvants. Therefore, modern vaccinology require harmless and effective adjuvants for the better immune system production. In recent years, tried to eliminate this drawback and developed various approaches. One of the techniques is development of polymeric nanoparticles which is completely

biodegradable and biocompatible in nature and having quality for better immunogenicity and stimulation of cellular as well as humoral immune system. It is more convenient and administered easily by nasal route but another vaccine like DNA-based vaccine is less used in human but mostly used for animal models, especially for spreadable disease. The newly developed polymeric nanovaccine works well in human immune system and cure the cells from foreign microorganisms.

2.10 Polymer-based vaccine

Polymer is used for development of polymer based nanovaccines delivery system. For this purpose, antigen or protein or peptide is encapsulated in polymeric shell. These proteins and peptides molecules are obtained and purified from microorganisms, otherwise developed a recombinant DNA technology using weakly antigenic substance or synthesized chemicals. This polymer based vaccine containing protein and peptide has effective immune stimulating property and has also practically proved that adjuvants given alone act as the immune stimulators or act as non-specifically important substance for increasing the immune-stimulating response of week antigens. Polymeric nanovaccine will be nonexpensive and more powerful than conventional vaccination. There are two types of polymeric nanoparticles, the first is self-assembling nanoparticles prepared from incorporation of modified adenoviruses which deliver at the target gene. Cells using this gene produce the protein, which increases the immune response. Another type of vaccine is using synthetic polysaccharide, which directly target to protein molecules, attached with special carbohydrate binding domain. The technology is only compatible for 50-90 nm diameter particles, this range of particles are easily recognized by cells [Harding and Song, 1994].

The available traditional vaccines are attenuated or heat-inactivated viruses, which causes many unwanted side effects. In comparison to traditional vaccines, subunit protein and peptide vaccines are safer and have well-defined components. But the drawback of subunit protein and peptide vaccine is poor immunogenic and require additional adjuvants for production of sufficient immune response. Therefore, selection of particular adjuvant is important for the development of subunit vaccine. An antigen entrapped polymeric nanoparticles represent a new approach to provide control and sustained release of antigen at the site and production of the desired immune response. According to literature, the biodegradable polymeric nanoparticles are good carrier for antigen delivery system and also provide systemic as well as mucosal vaccine delivery systems [Akagi and Akashi, 2006].

The polymeric nanoparticles are available in various sizes and shape with good mechanical properties. They are more biocompatible and biodegradable in nature, which is more valuable for the fields of tissue engineering, biomedical and pharmaceutical engineering. In immunological and vaccine development field, when antigen incorporated in polymeric shell, it acts an adjuvant. According to literature, an adjuvant is the substance, which is combined with viral or bacterial antigen to enhance the innate and adaptive immunological activity. When polymer is used as adjuvant it will be more compatible and immunologically inert with no significant toxicity. There are several advantages of polymer, when administered with an antigen:

- Antigen encapsulated polymeric nanoparticles shows sustained release, as like of depot carriers and modulate the cells immune response.
- An antigen integrated polymer follows various signalling pathways, for example when antigen loaded polymeric nanoparticles enter the body, it can be

phagocytosed and processed by protein complexes proteasomes. After that, inflammatory pathway is activated and secretes of IL-1b cytokine. Also releases various cytokines that help and enhance the production as well as interaction of T and B cells. Activation of immune response involve many cytokines, chemokines, proteases and effector cells like macrophages, mast cells, osteoclasts, neutrophils and eosinophils [Nanda and Holmdahl, 2006].

- Loaded polymeric nanoparticles shows sustained or slow release of antigen.
- The mycobacteria encapsulated in polymeric adjuvant shell interact with the pathogen or bacterial recognition receptor like toll-like receptors (TLRs), which enhance the T cells proliferation and activation [Freund, 1956].
- Polymers are capable to work like adjuvants and can be used in place of the conventional adjuvants, due to their excellent biocompatible and biodegradable nature. The first polymer which is used for the activation of immune response is LPS. It is effective, and can be administered with an antigen [Louis and Lambert, 1979]. The naturally occurring polymers b-glucans and zymosan are isolated from microbes, which have excellent adjuvant properties and are useful for the development of autoimmunity [Hida *et al.,* 2005; Hida *et al.,* 2006].

2.11 Delivery of therapeutic molecules

The effective delivery of the therapeutic molecules requires easy crossing across the barrier, reaching the target site and fighting against the disease causing agents. Many currently available drugs have shown good response at the target site but most of them have chances of causing toxicity and cell impermeability. Selected drugs can be either entrapped, encapsulated, adsorbed or dissolved into nano sized specific matrix system to decrease the harmful side effects, reduce degradation of drug, increase bioavailability

and enhanced drug targeting. Polymeric nanoparticle system is more beneficial and effective in comparison to traditional system in following ways:

- Nano size of particles could improve degradation as well as diffusion and shows time dependent controlled release of drug by the diffusion of encapsulated materials or degradation of outer shell.
- Nanometric sizes of particles influence the rate of absorption, distribution, metabolism, and excretion.
- Polymeric nanoparticles may allow binding drug at target receptor site and influences the receptor activity, for example, delivery of porcine somatotropin (pST) nano encapsulated in co-polymer PLGA (poly D, L-lactide-co-glycolide) in a time-controlled release manner to swine [Leoni and Desai, 2004].
- It is a valuable tool for the treatment of enzyme or hormone related disease, when encapsulated cells are given to the body.
- Another example of the polymeric nanoparticles is the establishment of new immune system, such as pancreatic cells of rat is encapsulated and allowed to secrete insulin through the pores, and protected the immune responses at the same time [Leoni and Desai, 2004].
- Various bactericidal and viricidal topical products for humans and animals are also prepared by nanotechnology process. It successfully delivers the drugs against the protozoan, fungal, viral and bacterial diseases like leishmaniasis, tuberculosis, salmonellosis and candidiasis *etc*. The development of lipid based nano formulation of amphotericin B has shown broad spectrum activity for fungal infection [Chu and Sadullah, 2009].
- Several studies suggested that nanoformulations have greater efficiency and safety in comparison to conventional products.

- Nano formulation is easy to formulate and capable to treat various diseases or infections.
- Nanoparticles work as smart delivery system *i.e*. it is having the capability for to programmed detection, self-regulation and delivery of therapeutic molecules at the target site. It also monitors the effects of delivered molecules, pharmaceutical drugs, food supplements, chemicals, nutraceuticals and vaccines [Scott, 2007]. Super paramagnetic nanoparticles, nanoshells and quantum dots have also been used to identify, trace and destroy infectious organisms or diseased cells [Scott, 2005].

2.12 Polymeric nanoparticles for antigen delivery and adjuvants

Development of new vaccine for cancer or other infectious disease is valuable for generation of effective adaptive immune response. Available conventional marketed vaccines are either attenuated or live or bacterial toxins or whole virus pathogens, but this type of developed vaccines has been useful only for specific antibody production and destroy the antigen though cytotoxic T lymphocyte (CTL). For the long lasting immunity, T cells play an important role because it regulates both cellular and humoral immunity. When foreign antigen enters into the body, immune systems immediately recognize and generate immune response. If this immunity is due to involvement of T-cells, immunological T-cells memory is generated. When body encounters the same antigen it produces stronger immune response due to immunological memory. The principle of the immunization is priming and booster, priming is the first antigenic stimuli, whereas booster is the subsequent antigenic stimuli [Zhao and Leong, 1996; Singh, 2002].

Vaccines developed in ancient times directly target the dendritic cells (DC) and produce prolonged and sustained release (Figure 2.7). Polymeric nanoparticles are up taken and processed by dendritic cells, therefore it achieved prolonged activity. Dendritic cells play essential role in development of better immunity and specific responses especially like T-cell immune response generation, which is most effective in antigen-presenting cells (APCs). It also regulates some extensive area of adaptive immune system by internalization process [Banchereau and Steinman, 1998].

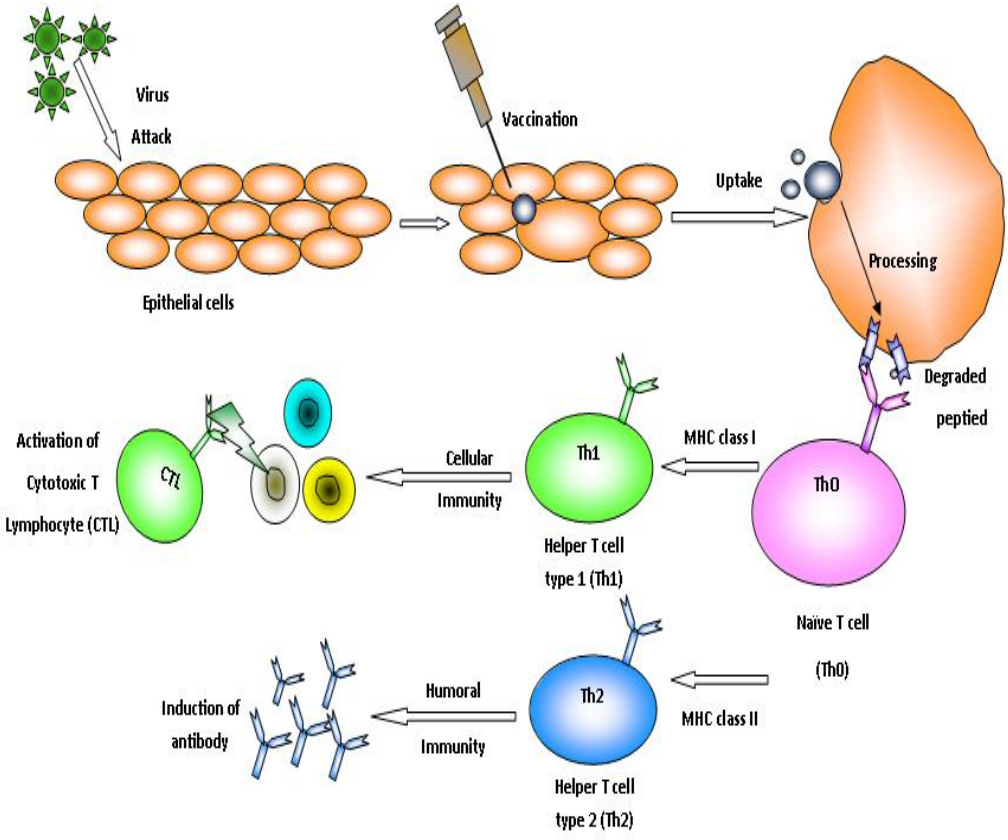

Figure 2.7: Schematic representation of polymeric nanovaccine administration. Nanovaccine shows uptake internalization, activation of MHC class I and cellular immunity and finally production of CTL. Another pathway is activation of MHC class II and humoral immunity and finally production of antibody

The polymeric coating of antigen is not influenced the uptake by dendritic cells. The adjuvant properties of polymer shows significantly uptake into DCs. It is also observed that submicron size of polymeric nanoparticles have more advantages compared to microparticles. Nano size polymeric particles showed greater intracellular uptake compared to microparticles [Foged *et al.,* 2005; Kanchan and Panda, 2007]. Adjuvants are described "substances used in combination with a specific antigen that produced a more robust immune response than the antigen alone". The well approved licensed adjuvants for human are phosphate salts and hydroxide of aluminum and calcium. Alum type of adjuvants showed many disadvantages, when incorporated with vaccines [Brewer, 2006].

In place of alum vaccine, antigen loaded nanoparticles gave better results for antigen specific selective targeting and improvement of humoral as well as cellular immune response. The potential of the adjuvants depends on the degree of dendritic cells maturation, when they come in contact with liposomes or polymeric nanoparticles. Maturation of DCs increased many cells surface markers and also stimulated the molecules like CD40, CD80, CD83, CD86, MHC class I, and MHC class II. It also induced the inflammatory marker such as bacterial DNA, lipopolysaccharide (LPS) other cytokines inflammatory markers such as IFN-γ and TNF-α [Reddy *et al.,* 2006, Babensee, 2007; Black *et al.,* 2010].

2.13 Distribution of nanoparticles

Any particle when enters in the body, firstly internalized by endocytosis process. The process of endocytosis mainly depends on particles size, shape, and surface charge. After endocytosis, the internalized materials finally depredated and transferred by endosomes to lysosomes. Hence, degraded exogenous antigens further followed the

MHC class II pathway and this pathway is involved in antigen antibody-mediated immune responses. While existing antigens by MHC class I pathway, generated cytotoxic T-lymphocyte (CTL) response [O'Hagan, 1998; Storni *et al.*, 2005; Shen *et al.*, 2006].

The prevention of antigens internalization by endosomes is a significant issue in control of antigen presentation or processing pathways. For successful release of polymeric nanoparticles from endosomes, membrane disruptive agents are required to release internalized polymeric nanoparticles into the cytoplasm. Some agents which help in membrane-penetration such as pathogen derived pore-forming proteins, some peptide, lipid or polymer which disrupts the endosomal membrane (endosome escaping) and reduced the pH inside the cells compartment. Therefore, in recent year various pH sensitive polymeric nanoparticles have been developed for enhancement of cytoplasmic delivery [Plank *et al.*, 1998; Shai, 1999; Yessine and Leroux, 2004].

The osmotic colloidal mechanism also plays a significant role for the degradation of polymeric nanoparticles. It quickly degrades into several smaller fragments. Increase in osmotic pressure of endosomes causes immediate entry of water through the membrane and causes disruption of the membrane. Some research articles proved that the acid-sensitive nanoparticles induced antigen-specific response and produced showed antitumor activity [Murthy *et al.*, 2003; Chen *et al.*, 2005]. Polycations are also responsible for the acidification of endosomes which break these vesicles. The mechanism involved is absorption of protons by acid organelles and generation of osmotic pressure, which results in swelling and rapture of endosomes, and release of internalized materials into the cell cytoplasm [Standley *et al.*, 2004]. Mostly polymer contains hydrophobic alkyl and carboxyl groups and present protonated ions at internal

endosomal pH. At low pH, hydrophobicity gets increased and polymers can easily enter into the endosomal membranes and cause disruption [Boussif *et al.,* 1995].

Polymer-based particles are promising vaccine adjuvants due to their synthetic addressability, tunability and ability to perform multiple functions necessary for successful generation of an adaptive immune response. On the most basic level, particulate matter is efficiently and selectively taken up by phagocytic cells, which provides a basis for targeting APCs in the body. Beyond this, polymer-based antigen delivery systems can be engineered to interact with APCs in defined ways to produce specific immune responses [Panyam *et al.,* 2002]. There are obviously a large number of challenges and parameters to be considered while developing polymeric subunit vaccines. These new formulations should ideally have the following criteria:

(1) Induce an appropriate immune response for the disease of interest,

(2) Protect antigen from premature degradation before delivery to appropriate APCs,

(3) Induce immunity after minimal number of doses,

(4) Cost effective to produce and transport,

(5) Reduction in unwanted side effects or illness, and

(6) It composed of biocompatible/biodegradable materials that can be readily cleared from the body.

2.14 Aspects of immune response

HLA-linkage: Human immune system is very vast system that will destroy and remove foreign invaders. Immune system having human leukocyte antigen (HLA) is coded by the major histocompatibility complex (MHC) group. It is present in six chromosome of the human genome. The human leukocyte antigen is composed of three classes' of molecules, in which class I and II are all time active for immune response *i.e.* involved in immune pathogen reaction. Mainly HLA class I molecules are found in cells nucleotide and play important role on defence against the various pathogen inside the cells. The human leukocyte antigen class II molecules found on surface of antigen presenting cells (APCs). Mainly macrophages, dendritic cells and B-lymphocytes are under the class II HLA. The human leukocyte antigen class II molecules contain two α and β chains, containing two domains [Lango and Lindblom, 1993; Trachtenberg *et al.,* 2007; Begovich *et al.,* 2001]. Finally, both class I and class II human leukocyte are involved in the immune response generation and defence mechanism against the Hepatitis B antigen [Milich and Leroux-Roels, 2003].

Antigen presentation: The main function of the antigen-presenting cells (APCs) in immune system is ingestion of foreign protein, especially in case of HBsAg. It degrad the peptides and break into 12-15 amino acids. Inside the cell, class II human leukocyte antigen is linked with degraded viral peptides and formed the association complex. These complexes are transported and associate to the surface of the APCs. The peptide-loaded HLA class II molecule also is present in lymph nodes of APCs surface. The APC is recognized by T cell receptor (TCR) of HBsAg-specific CD4+ cells.

These activated T cells in turn activate HBsAg-specific B cells, situated on surface of same human leukocyte antigen class II molecules. Subsequently, B cells mature and

generate heavy chain expression of the secreted immunoglobulin. After the maturating of plasma B cell, it proliferates and produces IgG subclasses in blood [Borzi *et al.,* 1992; Shokrgozar and Shokri, 2002; Wang *et al.,* 2005). Apart from these, T helper cells, T memory cells and B memory cells are also play significant role in generation of immune memory in case of Hepatitis B vaccination as well as in natural Hepatitis B infection [Banatvala *et al.,* 2000].

2.15 Nanoparticles: advantages and disadvantages

When antigen is encapsulated in polymeric shell attach by covalent bond, the covalently bonded nanoparticles offers the following advantages:

A low dose of antigen is sufficient for the better immune responses and easily stimulates the antigen-presenting cells for further processing. It shows good stability when kept in long time [Gengoux and Leclerc, 1995]. Nanoparticles of antigens show better immunogenicity and sustained release due to absence of adjuvants. The most widely used adjuvant in conventional Hepatitis B vaccine is alum, but it is well known to cause irritation. The needle free immunization using nasal administration of nanoemulsion of Hepatitis B vaccine has given good effectiveness as well as tolerability and causes no inflammation. The stability of the nanoformulated Hepatitis B vaccine is good compared to conventional vaccine. It does not require refrigeration, stable at 25°C for a month and 6 weeks at 40°C, so that it can be easily to transported from one place to another.

Now days, number of vaccines are available in market for various diseases but most of the vaccine are high cost, require multiple dose of administration, low immunogenic activity and needs cold storage. For the replacement of conventional vaccine,

developing nano/microparticles of various polymer and lipids that are biodegradable and very biocompatible in nature is essential for time dependent control release and degradation. The nano/microparticles show higher immunogenic properties and stimulate the cellular as well as humoral immunity [Kersten *et al.,* 2004]. The water-in-oil-in water emulsion solvent extraction or evaporation is the most common method for formulation of antigen loaded nano/microparticles. In this process, primary emulsion (W/O) containing antigen in aqueous phase and polymer in organic phase is homogenized by homogenizer. The prepared primary emulsion is again homogenized with aqueous phase. The drawback of this technique is reproducibility of the fabrication of nanosize particles.

Another problem is aggregation of the particles [Patel *et al.,* 2008; Kalkanidis *et al.,* 2006]. Certain physical parameter influence the formulation of polymeric nanoparticles, like entrapment efficiency, release of antigen though polymeric shell, morphology of particles, size distribution and zeta potential. It can be controlled by using appropriate amount of the polymer in formulation. These types of prepared nanovaccine are non-invasive, therefore administered by various routes like oral, nasal or in the form of diffusion patches or microneedle arrays. Hence, reduction in pain and minimal damage to skin may be possible. Other benefits are elimination of multiple injections, as one dose of vaccine is sufficient for the complete immunization [Kendall, 2006]. Polymeric nanoparticles are stable, safe, pure and is easily sterilized by (gamma radiation) non-thermal methods. It has been reported that the nano or micro polymeric HBsAg loaded particles showed different immunogenic activity [Sharma *et al.,* 2009; Fifi *et al.,* 2004]. Immunogenic activity of the nanoparticles is more in comparison to micro particles. Chen's group investigated that, as size and shape of the particles is changed, reduction

in toxicity was observed because nanoparticle gets quickly cleared from the body due to smaller size [Zhang *et al.*, 2006; Service *et al.*, 2008].

2.16 Route of administration of Hepatitis B vaccine

Due to the smaller size and large surface area, the nanomaterial/nanovaccine can easily enter the cell, so that the use of nanomaterials is significantly increased and used in various biological as well as other fields of application. The nanotechnology provides concept of minimizing the side-effects and depositing the drugs/therapeutic molecules at the desired site. For increasing the activity of nono formulation, different route of delivery is available. The following are different routes of administration and is described in Figure 2.8.

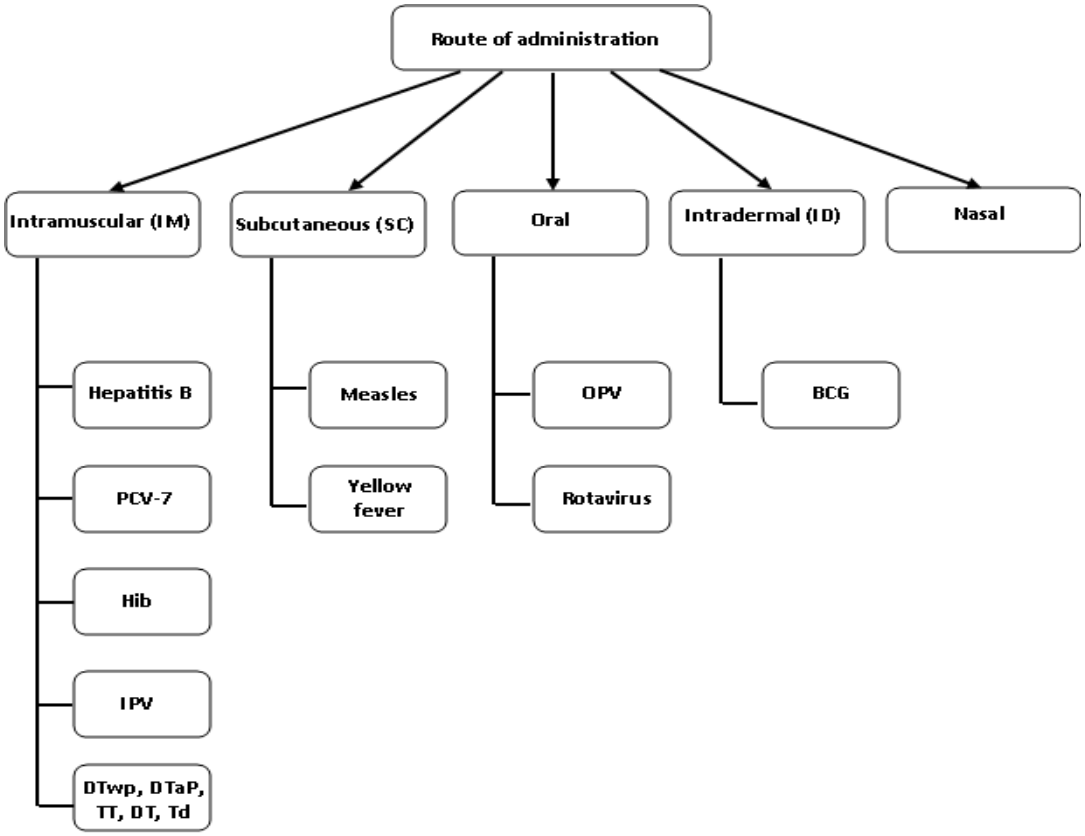

Figure 2.8: Different routes of administration (intramuscular, subcutaneous, oral, intradermal and nasal) of various vaccines

2.16.1 Oral Route

This is one of the most common and easy route of administration**.** Concentration of any type of substance like to peptide/ protein antigen gets diluted through this route. Higher concentration may be required for its efficacy as on passage through the gastro-intestinal tract the concentration gets diluted. Nanotechnology based polymeric particles are used for the oral DNA vaccines [Bhavsar and Aniji, 2007]. DNA vaccines are diluted and actively transported at the gastrointestinal tract, but for the better effectiveness more concentration is required. These are the major disadvantage of the oral route of administration that requires higher amount of the drug for complete action.

2.16.2 Nasal route

This route of vaccine targeting is developed because the conventional intra dermal vaccine causes many problems like irritation, pain and redness. Side effects are due to the presence of alum as adjuvant is the major limitation. To remove this drawback, Hepatitis B antigen vaccine is prepared using soyabean oil, chloroform, acetone, water and emulsifier agents through nanoemulsion technique.

The delivery devices for the administration of nasal vaccine are disposable devices, small in size and deliver the accurate amount of therapeutic agents. At present limited number of nasal sprays are available in market like Flumist for influenza virus. New type of nasal spray is bidirectional nasal delivery device which delivers drugs at posterior connection between the nasal passages. Other advantages of bidirectional nasal delivery device is less the deposition of particles in lungs and easy to handle, but conventional inhalation having high risk and problem of particles deposition. The bidirectional nasal devices for influenza and diphtheria virus vaccines are available in

market which gives favourable results [Djupesland *et al.,* 2006]. Actual problems with the nasal delivery of antigens are:

1. The free antigen is immediately removed from nasal passage.

2. Nasal epithelial layer shows very poor absorption.

3. Comparatively low immune response.

To overcome the drawback of direct nasal drug delivery system, encapsulated or adsorbed bioactive nanoparticles can be developed. It gives better antigen targeting and maximum the immune activity [Slutter B *et al.,* 2008].

2.16.3 Intradermal route

This route is general and most widely used route of vaccination. The dose is injected in the outer layer of skin to reach epidermis, which is immunogenically sensitive layer [Bal *et al.,* 2010]. Nanoparticles on administration by intradermal route increase the immune response. It is observed with mixture of antigen/TMC incorporated THC nanoparticles. In this, TMC act as immunepotentiator, helps in skin penetration and delivers the antigen. Most of the nanoparticles show maximum immunogenic activity in nasal and intradermal routes of administration.

2.16.4 Microneedle Patches

In the conventional method, the delivery of vaccine through the intradermal injection of the antigen, but the antigen may not reach the Langerhans cells of the epidermis to induce immune response. Intradermal route is pain full, irritating and produce redness and swelling. To overcome these problems, a microneedle patch was developed that can

directly applied to the skin. The molecules less than 500 Da can be easily delivered in this route. Plasmid DNA or protein or antigen is coated on the surface of nanoscale tips *i.e.* adhered on microscale length needles. Another advantage is effective and safe delivery of the biomolecules to epidermis layer of skin [Chen *et al.,* 2008]. Use of microneedle patches for vaccination against influenza has been reported [Pearton *et al.,* 2010].

2.17 Mouse models for Hepatitis B

The development of the animal models for Hepatitis B virus infection is very difficult due to narrow host range, as it mainly infects human. Experimental infection model of chimpanzees, tupaia and asian tree shrew have been used [Dandri *et al.,* 2005; Walter *et al.,* 1996]. The most immunocompetent host is chimpanzee which is fully susceptible to Hepatitis B virus infection. When infected human Hepatitis B virus serum is injected in chimpanzee, it showed induction of acute hepatitis [Barker *et al.,* 1973]. But it is restricted for research of human hepatotropic viruses and difficult to perform experiment due to their large size, high cost and require strong ethical constraints. Tupaias is another option for Hepatitis B virus infection studies but it shows mild infection and has short life viral titers. HBsAg secrete in blood serum and generation of antibodies against to HBsAg and HBeAg [Walter *et al.,* 1996]. Another drawback of tupaias is availability, not easily found in every place, difficult to handle due to relatively large size.

Thus, due to restriction of suitable animals model, it become very important to develop a new and well defined small animal mice model of human animal chimeras called as humanized or xenograft mice. It provides an important tool for implantation of human substance in animal mice model. These models give an opportunity to basic and clinical

studies of pathophysiology of Hepatitis B virus. The following are the list of Hepatitis B virus small animal mouse models:

1. HBV-transgenic mouse model
2. HBV-transfection by hydrodynamic injection model
3. HBV-transfection through adeno-associated virus model
4. Genetically humanized mice model
5. Humanized mice with human immune system and liver tissue model
6. Chimeric mouse model

Table 2.3: Comparatively features of various mice models

S. No.	Mouse Models	Advantage	Disadvantage
1.	HBV-transgenic mouse model	Useful for virology study due to very-high-level HBV replication	Difficult to analysis the immune response and HBV infection pathophysiology.
		Immunologically tolerant for the virus	Due to tolerance no immunogenic response showed.
			Viral replication is not analyzable.
			Viral protein transcripts from the integrated transgene.
			Single viral encoded protein.

2.	HBV transfection by hydrodynamic injection model	Hepatitis B can be observed	Handling of full-length HBV genome is difficult.
		Immune response generated against to virus	Viral protein expression is observed but it is quickly dismissed within 15 days.
			Liver is not completely homogeneously infected by HBcAg.
3.	HBV transfection through adeno-associated virus	Core antigen (HBcAg) is expressed in this model	Handling of Adeno-Associated Virus is difficult
4.	Genetically humanized mice	Life of the mice is higher compared to another model	Hepatitis B virus entry is allowed but it is not supported for other viral infections
			Hepatitis B virus infection is limited
5.	Humanized mice with human immune system and liver tissue model	T cells response is clearly observed against the virus	Very difficult process for development of mice model
		Models are appropriate for immunology and viral infection study	The life of the mice model is low
6.	Chimeric mouse models of HBV infection	Complete viral infection viz. infection, entry, replication and packaging can be studied	Development of mice model is difficult
		Clinical specimen and suitable models for Hepatitis B	
		Useful for the testing of the anti-HBV agents	
		Immune response and HBV infection pathophysiology can be studied	
