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SEROLOGICAL MARKERS OF HBV INFECTIVITY

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Summary. - *The infectivity of a serum sample for hepatitis B virus (HBV) can be excluded only when it is inoculated intravenously in susceptible hosts (chimpanzees) without inducing seroconversion to markers of HBV. Other tests can be used as surrogate markers of HBV infectivity. Screening of blood for hepatitis B surface antigen (HBsAg) had a tremendous impact in the reduction of post transfusion type B hepatitis. The absence of anti-HBc, HBsAg and HBV-DNA in a given serum would almost completely exclude HBV infectivity, however the use of all these tests together is not always worthed in terms of cost/benefit and we have to select one of them according to the different goals and situations. Detection of HBV-DNA by molecular hybridization is a simple, sensitive and reproducible procedure that allows rapid examination of multiple samples. Detection of viral nucleic acid can not be proposed as a routine test for blood screening because the finding of HBV-DNA in a HBsAg negative serum is only a remote possibility. A very sensitive hybridization assay, howeve, should be mandatory to test biological material (immunoglobulins, clotting factors or other blood products) processed for use in man. Detection of anti-HBc as screening test of blood units is worthless in countries like Italy where more than 25% of the general population have serum markers of HBV infection. In conclusion we are almost (> 99%) sure that a given biological sample is not infectious for HBV when it is repeatedly negative for HBV-DNA even if other markers of HBV can be present in it (HBsAg or any antibody to HBV). Of course we can not exclude the potential infectivity of a given person only because her/his boold is negative for HBV-DNA. Too many dynamic events occur in the natural history of HBV infection that no single test can ever explain.*

Riassunto (Marcatori sierologici per l'infettività da virus dell'epatite B). - *E' possibile escludere l'infettività per il virus dell'epatite B (HBV) di un dato campione di siero solo qualora sia inoculato per via parenterale in ospiti suscettibili (scimpanzè) e non induca sieroconversione ai marcatori dell'HBV. Esistono tests alternativi e tra questi lo screening del sangue per l'antigene di superficie del virus dell'epatite B (HBsAg) gioca un ruolo fondamentale nella prevenzione delle epatiti post-trasfusionali di tipo B. L'assenza di anti-HBc, HBsAg, HBV-DNA in un dato siero permette di escludere completamente l'infettività, tuttavia l'impiego combinato di questi tests non è sempre vantaggioso in termini di costo/beneficio, e si rende pertanto necessaria la scelta di uno di essi in relazione ai differenti scopi e alle diverse situazioni. La determinazione dell'HBV-DNA mediante ibridizzazione molecolare è una procedura semplice, sensibile e riproducibile che consente una rapida determinazione anche di molti campioni. Tuttavia la determinazione dell'HBV-DNA in un siero negativo per HBsAg è una possibilità così remota che questo test non può essere proposto come saggio di screening per il sangue. Un test di ibridizzazione molto sensibile dovrebbe tuttavia essere impiegato per saggiare materiali biologici (immunoglobuline, fattori di coagualzione, e altri derivati del sangue) per uso umano. La determinazione dell'anti-HBc come test di screening delle unità di sangue risulta svantaggioso in paesi come l'Italia in cui più del 25% della popolazione presenta marcatori per l'infezione sostenuta da HBV. In conclusione è possibile essere quasi sicuri (> 99%) che un dato campione biologico non sia infettivo per HBV quando risulti essere ripetutamente negativo per HBV-DNA, persino in presenza di altri marcatori dell'HBV (HBsAg o anticorpi rivolti verso l'HBV). Ovviamente non è possibile escludere la potenziale infettività di un soggetto solo perchè il suo sangue risulta negativo per HBV-DNA. Gli eventi dinamici che si susseguono nella storia naturale dell'infezione da HBV sono troppi per essere interpretati con un solo test.*

Introduction

The measurement of infectivity of hepatitis B virus (HBV) in a biological specimen is a difficult task. Infectivity can only be exluded when this sample inoculated intravenously in susceptible hosts (chimpanzees) does not induce seroconversion to markers of HBV. Multiple

inoculations, however, are required to exclude a natural occurring resistance in some of these animals even in absence of serological markers of previous exposure to HBV. The cost of these experiments limits their use to test vaccine preparations and can not be proposed in other circumstances. Other tests are therefore used as surrogate markers of HBV infectivity and the screening of blood for hepatitis B surface antigen (HBsAg) has a tremendous impact in the reduction of post transfusion type B hepatitis. To understand the importance of serum markers of HBV infection as index of infectivity it is necessary to remind their biological significance and occurrence in the natural history of HBV infection. In this work we summarise the current knowledge of markers of HBV infection and discuss their use as indexes of infectivity.

Hepatitis B virus markers

Hepatitis B virus (HBV) belongs to the group of *Hepadna* (hepatitis DNA) *viridae* which are hepatotropic agents with a DNA genome [1]. The other well known members of this group are the woodchuck hepatitis virus (WHV), the beechey ground squirrel hepatitis virus (GSHV) and the Pekin duck hepatitis B virus (DHBV) [1]. HBV may infect its natural host (man) without pathological consequences or cause a highly polymorphic range of liver disease from inapparent hepatitis to cirrhosis. HBV plays also a role in the genesis of hepatocellular carcinoma.

One characteristic feature of hepadna viruses is to enter the hepatocyte then drive the synthetic apparatus of the cell to overproduce their structural proteins so that not enough genomes are packaged within such redundant material. The envelope of HBV (HBsAg), not assembled in the virion is released in the blood as free lipoprotein and circulates in spherical or filamentous particles of 22 nm in diameter. These particles do not contain nucleic acid and are not infectious; they represent the previously named Australia antigen. The excess of nucleocapsidic protein circulates as soluble antigen (hepatitis B e antigen, HBeAg).

The virion contains a small, circular, partly double stranded DNA molecule of 2.1 x 1,000 kDa of molecular weight [2]. It is formed by a long strand (L, minus -) of about 3,200 nucleotides and a short strand (S, plus +) of variable length, 50-100% of the complete L (-) strand (Fig. 1) [2]. The circular structure is maintained by base-pairing of about 260 nucleotides at the 5' end of the two strands. Sequence analysis of HBV-DNA reveals 4 major open reading frames (ORFs) on the L (-) strand: they are defined S, C, P and X. The L (-) strand RNA transcript codes for at least 4 proteins. No ORF of significant length is present on the S (-) strand. At both sides of the cohesive ends of the two strands there are two copies of an 11 base-pairs (5' TTCACCTCTGC) sequence termed direct repeats, DR1 and DR2. A similar organization of the genome including the major ORFs and DRs is

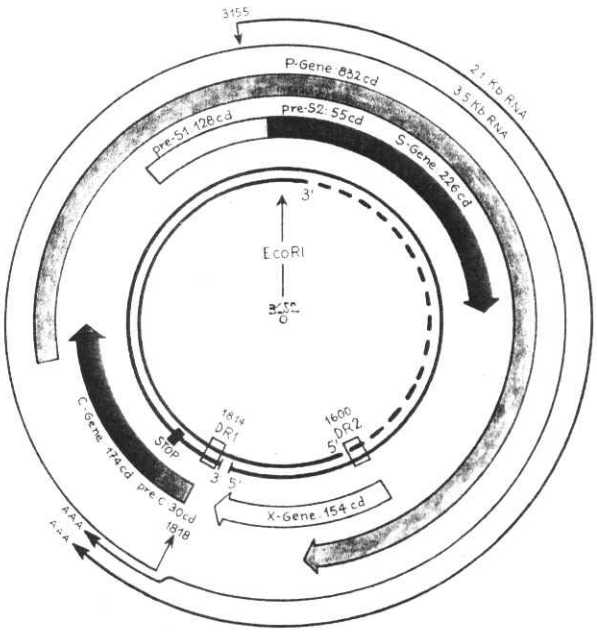

Fig. 1. - HBV genome.

conserved in all the subtypes of HBV and in the other hepadna viruses suggesting an important biological role of these sequences [3]. The S region codes for the protein of the viral envelope. HBsAg is conformational antigen composed of three proteins pairs which are co-terminal. The part common to all three proteins is coded by the S gene, a section of 55 aminoacids upstream is coded by the pre-S2 region and an additional section of 108-116 aminoacids is coded by the pre-S1 region (Fig. 1). Each of the three possible proteins is facultatively glycosylated in its S gene part thus six HBs proteins are found by gel electrophoresis. P24s and its glycosylated derivate GP27s are the major proteins. GP33s and GP36s contain in addition the pre-S2 sequence and are always glycosylated. P39s and GP42s contain both pre-S1 and pre-S2 regions and the second of these proteins is glycosylated. HBV and HBsAg particles from highly viremic carriers contain more GP33/ 36s than HBsAg particles from carriers with low titer of HBV in the blood. Complete HB virion particles contain also much more of the two larger HBs protein, P39s and GP42s. In contrast the 22 nm HBsAg particles contain only traces of this protein pair.

The C gene encodes the nucleocapsidic protein. Two AUG initiation sequences are present at positions 1814 and 1901 in the genome's map identifing a pre-C and C region (Fig. 1). Initiation of translation at the pre-C codon would produce a hydrophobic polypeptide of 30 aminoacids representing the leader sequence of the native nucleocapsidic protein [4]. The product of pre-C and C regions (P25e) is translocated through the endoplasmic reticulum's (ER) membrane then the leader protein is cleaved off and the rest of the protein remains anchored to the membrane. Some of these peptides are used to build the "core" particle, the nucleocapsid of HBV. The

protein that is not assembled in the nucleocapsidic particle is cleaved by a trypsin like protease of the infected cell and secreted in the blood as soluble antigen (P16e or hepatitis B "e" antigen, HBeAg).

The X region can code for a peptide of about 150 aminoacids. The function of this region remains unknown but antibodies against the translation products of this gene have been found more frequently in HBsAg positive sera of patients with hepatocellular carcinoma [5].

The translation product of the P region is a basic protein, rich in histidine, of about 90,000 MW (mosaic virus) representing the DNA polymerase of HBV. Interestingly there are tracts of homology between the aminoacid sequence of this protein and that of the reverse transcriptase of retroviruses and cauliflower mosaic virus (CaMV) [6]. This evidence suggests a potential reverse transcriptase activity of HBV DNA polymerase. This hypothesis is in keeping with the replication strategy of HBV where reverse transcription of pregenomic RNA forms in genomic DNA is cardinal for viral multiplication. Hepatitis B virus synthesizes its open circular DNA genome by reverse transcription; a RNA copy of the DNA genome (L or minus strand) is used as intermediate in the replication cicle [7]. Thus the virus enters into the cell, is uncoated and its genome moves to the nucleus where it is converted to complete open circular double-stranded DNA and to supercoiled DNA. The minus (L) strand is transcribed by the cellular RNA polymerase II to multiple forms of 3.5 kb pre-genome RNAs. The synthesis of the first DNA strand (L, minus strand) is initiated within the DR1 sequence and is primed by a protein which remains bound to the 5' end of this strand. The synthesis of the second DNA strand (S, plus strand) would be primed by a oligoribonucleotide remnant of the pregenomic RNA strand degraded by an RNase H-like activity [8]. This small RNA fragment of about 20 bases derives from the 5' end of the pregenome and contains also the DR1 sequence. Because the plus strand begins with a sequence adiacent to DR2 the putative RNA primer would be transposed to the position of DR2 before initiation of plus strand synthesis.

Two major poly (A) + messanger RNAs (mRNA) are seen in infected cells; both are minus strand transcripts and are referred to as the 2.1-kilobases (kb) and the 3.5-kb mRNA. The 5' end of the 2.1-kb mRNA maps about 20 bases upstream of the pre-S2 region and the 3' end to the beginning of the C gene (Fig. 1). This m RNA would encode pre-S2, S and X gene products. The 5' end of the 3.5-kb mRNA maps at the beginning of the pre-C region and the 3' end at the same position of the 2.1-kb mRNA. This mRNA encode all gene products of HBV. No major splicing events seem to occur in HBV-DNA transcription.

The mechanism of integration of HBV-DNA remains unclear. There is no evidence that a unique or preferential site is used for integration [9]. This appears to occur rather randomly and can be associated with extensive rearrangement of cellular DNA and partial deletion of viral sequences. No correlation between HBV-DNA integration and expression of known oncogenes was found. Dejean *et al.* [10], however, have reported the existence of a virus specific site of integration through the DR sequences. Thus integration at the DR site stops transcription of the 3.5-kb mRNA as its initiation sequence is adjacent to DR1; transcription of 2.1-kb mRNA is maintained. This implies that in cells where integration occurs C and P genes are not expressed and synthesis of viral proteins is limited to those specified by the 2.1-kb mRNA.

Serological markers of HBV and HBV infection

The mechanism of HBV infection is not yet completely understood. The virus can be found in white blood cells of infected individuals suggesting that its replication in these cells may precede the infection of the liver [11].

A current hypothesis holds that the hepatocellular tropism of HBV is mediated by expression on the liver cell membrane of receptors that bind human polymerised albumin. A similar receptor is present on the HBV particle and it is specified by the pre-S2 segment of the S gene. However, another virus of the Hepadna family, WHV, does not show any binding property for polymerized albumin nevertheless it enters the hepatocyte as efficiently as HBV [12]. Thus the mechanisms of virus-cell interaction are poorly understood and probably multiple.

Following binding, the virus penetrates the cell by endocytosis, it is uncoated and its genome reaches the nucleus. HBV-DNA replicates in a unique fashion transcribing its DNA genome from an RNA intermediate then HBV-DNA binds specifically the nucleocapsidic peptides, is duplicated in a partially double stranded form and encapsidated. After assembly, the progeny of new viruses budd through the cell membrane and infection spreads to neighbour cells.

HBV is not directly cytopathic but expression of virus-encoded proteins on the cells surface renders the infected cell susceptible to immune attack and necrosis. The host immune response eliminates virus infection and causes liver damage. During the acute phase of viral infection complete HB virions, defective HBsAg particles and free HBeAg circulate in large quantities in the blood while liver function tests are normal. Liver damage occurs only when a strong immune response develops. Humoral response is predominantly directed against the structural proteins of the virus (HBcAg, HBeAg and HBsAg) while cell mediated immunity is directed against virus infected cells. The virus specific cell mediated response of cytotoxic T lymphocytes is restricted by antigenic determinants of the virus and those of class 1 of the major histocompatibility complex (MHC class 1) [13]. Hepatocytes can also express MHC 2 determinants on their membrane thus presenting viral antigens

to T cell for recognition. This entails that also non structural proteins of HBV synthesized during replication might elicit cellular immune response. Finally viral proteins on the cell membrane can bind circulating antibodies that modulate the intensity of the immune attack. The effector antigen of cell mediated response is believed to be the nucleocapsidic antigen whose peculiar feature is the antigenic duality, namely HBcAg and HBeAg. The two antigens are different epitopes of the product of one ORF. HBcAg corresponds to the 27 nm nucleocapsidic particle while HBeAg represents the excess of nucleocapsidic protein not assembled in the "core" particle and secreted as soluble antigen (see above). The large amount of HBeAg circulating in the blood distracts the host's immune system from attacking the hepatocytes that contain HBV and helps massive viremia. Disappearance of HBeAg followed by anti-HBe seroconversion is accompanied by flare up of liver disease. This occurs usually during the clinical phase of acute hepatitis as well as in chronic carriers of HBsAg with riactivation of liver disease [14]. The appearance and persistence of anti-HBe antibody in carriers of HBsAg who have lost active HBV replication is associated with recovery from liver disease but only the appearance of antibodies against viral surface antigen (anti-HBs) and particularly against the pre-S region encoded epitopes which neutralize specifically the virion herald the complete elimination of HBV [15]. Acute infection is generally benign and rarely it causes severe or fulminant hepatitis; this situation is rather frequent when HBV infection is associated with concurrent HDV or non-A, non-B infection. Persistence of HBV infection is more frequent in males than in females and in immunotolerant or immunodepressed hosts (as in newborn or hemodialised patients). Immunotolerance might be genetically determined as it is the immune response to HBsAg [16], however the evidence that HBV is able to infect and replicate in immunocompetent cells suggests a possible role of the virus itself in limiting the effectiveness of the immune response thereby favouring the chronic carrier state.

The natural history of chronic HBV infection is one of slow transition from the phase of active viral multiplication characterised by high levels of circulating HB virions to the phase of inactive infection where the virus exists in the integrated form in the host's genome and free viral nucleic acid is not detected in serum and liver [17]. Thus presence of HBeAg in serum is a reliable index of HBV infectivity but detection of HBV-DNA in serum by molecular hybridization has changed the conventional concept that active HBV replication is identified by presence of HBeAg while the homologous antibody (anti-HBe) corresponds to the inactive phase of infection [18]. High levels of viral DNA are usually present in serum of chronic HBeAg carriers and HBV-DNA disappears before or simultaneously with anti-HBe seroconversion. In a minority of patients, however, viral DNA may circulate for several months beyond clearance of HBeAg. Thus during the period of HBeAg/anti-HBe seroconversion a discrepancy between presence of HBV-DNA and HBeAg in serum is possible. The viral nucleic acid is infact a direct measure of the virion while detection of HBeAg is dependent on the relative excess of this antigen over the homologous antibody at the time of testing.

Another condition of discrepancy between HBV-DNA and HBeAg is the reactivation of viral replication in anti-HBe positive carriers of HBsAg. In this contest, however, presence of HBV-DNA in serum is associated or followed by reappearance of HBeAg. Free forms of viral nucleic acid were also found in serum and liver of a considerable proportion of anti-HBe carriers who have never been found HBeAg positive over time. Viral DNA was found in 50-70% of anti-HBe positive patients with liver disease but virtually in none of HBsAg carriers with normal liver. One possible interpretation is that HBV-DNA synthesis in presence of anti-HBe represents the late stage of the natural history of HBV infection following clearance of HBeAg [19]. Longitudinal studies of HBeAg carriers, however indicate that this may be the case of few patients [18]. Rarely viral DNA persists or reappears in serum after anti-HBe seroconversion and when this happens, it is for a limited period of time. HBV-DNA remains detectable in some anti-HBe positive carriers of HBsAg for many years suggesting that HBV replication is an integral component of their serological profile. The presence of HBV-DNA in serum of these patients is associated with a prevalent expression of HBcAg in the liver cell's membrane and cytoplasm opposite to the nuclear localization characteristic of HBeAg positive carriers of HBsAg.

The cytoplasmic staining of HBcAg correlates also with a severe histological lesion. These data are in keeping with the hypothesis that HBeAg represents the target of the host's immune response. The absence of serum HBeAg would not distract the immune system from attacking cells that contain replicating HBV and it might result in severe cellular damage. The larger amount of nucleocapsidic antigen bound to cell membranes of anti-HBe carriers with serum HBV-DNA suggests a defective maturation and/or secretion of this antigen. A defective proteolytic cleavage of P16e (serum HBeAg) from the native, membrane bound P25e might explain this finding. At least for two large families of viruses (paramyxovirus and myxovirus) the availability of specific host's protease and susceptibility of viral protein to cleavage are major pathogenetic determinants favouring spread of virus infection from cell to cell and eventually causing disease [20]. Alternatively a defective expression of the pre-C region of the C gene might hamper secretion of the nucleocapsidic protein as it happens for HBsAg when also the pre-S region of the S gene is expressed. These regulation properties of pre-S on HBsAg secretion were shown in transgenic mice where all somatic cells contained the whole S gene sequence integrated in their genome and additionally the pre-S1 region under control of an artificial promoter [21]. Synthesis of pre-S1 protein resulted in massive ac-

cumulation of HBsAg on the cell membrane without secretion of HBsAg in the blood. Expression of the S gene alone without induction of the artificial promoter of pre-S1 resulted in normal secretion of HBsAg. Finally another condition of discrepancy between absence of HBeAg and presence of HBV-DNA in serum is represented by carriers of HBsAg coinfected or superinfected with the hepatitis delta virus (HDV).

HDV is a defective hepatotropic RNA virus that requires helper functions provided by HBV. The virion is a spherical 36 nm particle with chimeric structure; it is coated by the surface antigen of HBV (HBsAg) and contains delta antigen (HDAg) and a RNA molecule of 1.7 kb (HDV-RNA) in the interior [22-23]. The entire HDV genome was recently sequenced and no significant homology was found between HDV and HBV genomes [24]. Delta particle contains two RNA species migrating in gel electrophoresis at 1.75 and 2.0 kb respectively [24]; these RNAs react only with one of the two strand specific probes of cDNA indicating that they have the same polarity (genomic) and may represent linear and circular forms of HDV-RNA [24]. These results and studies of HDV-RNA in the electron microscope suggest that HDV contains a single-stranded and covalently-closed, circular RNA molecule of 1678 nucleotides. Different forms of HDV-RNA were found in infected livers including genomic and antigenomic strands, poly-A RNA and double stranded forms (ds RNA). The presence of numerous ds HDV-RNA forms in the infected hepatocytes suggests a replicative cycle of HDV somewhat similar to that of satellites of plant RNA viruses. Satellite RNAs replicate in a rolling cycle fashion producing a large amount of ds RNA forms which accumulate in the cell. These forms suppress replication of the helper virus RNA that is produced much less rapidly than satellite RNA. In plants this event usually entails the attenuation of disease symptoms caused by the helper virus. Occasionally, however, superinfection with satellites containing a necrotizing gene causes tremendous epidemics of necrotising disease [25].

These features closely remind those of HDV infection suggesting a similar mechanism of inhibition for HBV replication induced by HDV. HBV-DNA was found only in serum of one third of the patients with acute HBV and HDV coinfection and in about one tenth of chronic carriers of HBsAg with HDV superinfection [22]. Another interesting evidence of inhibition of markers of HBV replication during HDV infection comes from biphasic hepatitis that predominates in drug addicts and is characterized by two peaks of serum transaminases (ALT). When a single inoculum or consecutive injections provide a material highly infectious for both HBV and HDV, the highly efficient synthetic apparatus of HBV supports the rapid rescue of the defective agent, which avidly takes advantage of the helper virus overwhelming the synthesis of HBV gene products and determining the first elevation of ALT. Subsequently, HBV replication completes its natural course, leading to the second bout of hepatitis corresponding to the elimination of both viruses. In keeping with this hypothesis is the evidence that HDAg is usually associated with the first ALT peak and it is followed by a strong anti-HD immune response at the time of the second ALT elevation. Since survival of the defective virus depends on the highly efficient use of the same replicative machinery of HBV, HDV may strongly compete with HBV and being an RNA virus may profit by the possible reverse transcriptase activity of HBV-DNA polymerase. Obviously, the inhibition exerted by HDV cannot be so intense as to suppress completely the synthesis of HBV, as some degree of replication of this virus is necessary for the survival of the defective agent.

In the HBsAg carrier inhibition of HBV replication exerted by HDV has important diagnostic implications. The decreased synthesis of HBV genes' products in carriers of HBsAg may herald either clinical deterioration secondary to HDV superinfection or spontaneous regression of HBV infection that usually leads to improvement of the clinical picture when HDV is absent.

When suppression of HBsAg is so profound as to result in the loss of the antigen from the serum, unrecognized carriers of HBV superinfected by HDV may present with negative serology and be diagnosed as having non-A, non-B hepatitis; this is likely to occur in individuals exposed simultaneously or sequentially to both viruses, as in the case, for example, of hemophiliacs and drug addicts. In these cases detection of HDV markers, anti-HBc and eventually HBV-DNA in serum is helpful to make a correct diagnosis.

Testing for HBV infectivity

The gold assay of HBV infectivity is the inoculation in susceptible animals but this is too expensive and can be proposed only in special occasions (vaccine preparation, etc.). Fortunately, as seen above we have a choice of other tests. The absence of anti-HBc, HBsAg and HBV-DNA in a given serum would almost completely exclude infectivity, however the use of all these tests together is not worthed in term of cost/benefit and we have to select one of them according to the different goals and situations. Detection of HBV-DNA by molecular hybridization is a simple, sensitive and reproducible procedure that allows rapid examination of multiple samples [18]. The quantity of HBV-DNA detected correlates with the infectivity of sera titered in chimpanzee, however the end point dilution of an infectious serum in the animal is still negative in this assay. Fractions of picograms (0.05-0.01 pg) of viral DNA, equivalent to a concentration of 10,000 virions, are determined by autoradioagraphy after a 24 h exposure and using ^{32}P labeled probes with specific activity higher than 1 x 10 dpm/μg of nucleic acid. Assuming that one virion corresponds at least to 1,000 defective HBsAg particles the extimated sensitivity of molecular hybridization is comparable to that of the most sensitive radioimmunoassays for detection of HBsAg. The relative sensitivity of the HBV-

DNA assay could be potentially higher in sera with a lower virion/HBsAg ratio. Detection of HBV-DNA in a HBsAg negative serum is such a remote possibility that detection of viral nucleic acid cannot be proposed as test of blood screening. A very sensitive hybridization assay however should be mandatory to test biological material (immunoglobulins, clotting factors or other blood products) processed for use in man.

Detection of anti-HBc as screening test of blood units is also worthless in countries as Italy where more than 25% of the general population have serum markers of HBV infection.

Detection of IgM anti-HBc was also proposed as a surrogate test for HBV infectivity, however presence of this antibody only in patients with liver disease and its absence in long lasting carriers of HBeAg/HBV-DNA with evidence of immunosuppression suggest that IgM anti-HBc is a marker of active host's reaction to HBV replication and only an indirect and a very unreliable marker of HBV infectivity [26].

In conclusion we are almost (> 99%) sure that a given biological sample is not infectious for HBV when it is repeatedly negative for HBV-DNA even if other markers of HBV can be present in it (HBsAg or any antibody to HBV). Of course we can not be sure that a person is not infectious when her or his blood is negative for HBV-DNA. Too many dynamic events occur in the natural history of HBV infection that no test of a single serum speciment can ever explain.

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