ISBT TTID WORKING PARTY
"HBV safety" study-group

Characterisation of "occult" HBV yield cases identified by ID-NAT (or small pool) or anti-HBc screening

Introduction

Hepatitis B and its causal agent hepatitis B virus (HBV) have been studied for many years both clinically and biologically. Yet a number of important questions remain unanswered. Individuals carrying the occult form of HBV infection defined as HBV DNA positive but HBsAg undetectable (1) is a missing link between clinically identified infection, recovery and the severe complications of the infection such as liver cirrhosis and hepatocellular carcinoma (HCC). Although classically these complications develop in individuals with signs of inflammation and/or fibrosis and evidence of hepatocyte lysis such as elevated ALT, some might develop in individuals totally escaping detection for many years such as carriers of occult infection.

The technological advances being made, such as the development of extremely sensitive nucleic acid detection and quantification systems systematically applied to large numbers of apparently healthy individuals such as candidate blood donors open for investigation a group of infected individuals that would have otherwise escaped medical scrutiny. These assays alone or combined with the long used anti-HBc assay are being increasingly applied to individual donation screening in areas with different epidemiology in terms of prevalence and genotype. Currently applied to a previously unscreened population the yield is going to be maximal until screened donors repeat donating (2, 3). This represents a unique opportunity of collecting large numbers of cases available for scientific and clinical investigation that will provide information on a nearly unknown part of HBV natural history. The proposal below will address issues not only from a blood safety point of view but also from a clinical and scientific point of view. It is the duty of the Transfusion community to explore the risks involved for donors and patients exposed to the so far uncovered population of occult carriers of a virus affecting globally close to a billion individuals.

Background

Blood screening

HBV screening in blood donations consists of HBsAg serological testing with assays varying in sensitivity from less than 0.1 to several nanogrammes/ml ranging from the fully automated highly sensitive PRISM to insensitive rapid tests such as particle agglutination. This screening is applied in areas with prevalence ranging from 1:5000 to 25% in populations where contact with the virus ranges from 0.2 to 100%. In addition, HBsAg screening assays were developed against HBV genotype A (prevalent in North America and North West Europe) while most infections are with genotype B and C in the Far East and South East Asia, genotype D in India, the Middle East, Eastern Europe, the Mediterranean basin and part of Africa, genotype E in West Africa and genotype F in native populations from Latin America (4, 5).

Anti-HBc was introduced in the USA as surrogate marker for non-A, non-B hepatitis but, since the availability of anti-HCV screening, it was maintained on the account of HBV safety although its exact efficacy remains under study (2, 6-9). This approach to improve HBV safety is however limited to areas where the prevalence is very low (<2%) otherwise the impact on donor deferral is considered unsustainable, in Southern Europe for instance and most of the rest of the world. The efficacy of the test is also limited by two main factors: its notorious lack of specificity and the very high proportion of samples containing high levels of anti-HBs (70->90%) making these blood units essentially safe for transfusion but, according to current procedures, excluded from the blood supply (6). This approach on its own leaves out two sources of potentially infectious blood: the pre-seroconversion window period and the tail-end cases of chronic infection that are anti-HBc negative-DNA positive (10).

HBV NAT has been introduced for several years in blood centres in Germany, Austria and Luxemburg but only in Japan is it utilised nationwide (11-13). In these areas and in specific studies conducted in the USA and West Africa for instance, HBV NAT was applied to plasma pools varying in size from 10 to 500 (2, 3, 13 -15). From some of these studies and from more recent unpublished data, it was clearly apparent that the yield of occult HBV was 3-10 times lower when tested in pools compared to individual donation samples (ID) (2, 3). In the context of the proposed study, ID screening with highly sensitive NAT is critical to study anti-HBc yield

samples where this approach is in use and everywhere else where NAT is the only possible blood screening approach.

Occult HBV and transfusion safety

At the present time, there is virtually no data on two critical issues relevant to transfusion: i) what is the infectivity of blood units or components containing occult HBV DNA with or without anti-HBc or anti-HBs outside the window period. In the window period, as little as 10 viral particles seem to be infectious. In contrast, in occult HBV carriers the Dane particles are often immune-complexed with neutralizing anti-HBs or other antibodies not necessarily detectable and presumably less infectious (16, 17). ii) What is the significance for the donor of being deferred on the basis of occult HBV in terms of possibility of transmitting the infection to others or the potential outcome of infection (1). The former is directly linked with the general issues related to the natural history of the infection discussed in the next chapter. Methodological approaches to answer these questions are limited and all are to some extent unsatisfactory since prospective studies are not ethically feasible and animal or in vitro experiments are not necessarily applicable to humans. The main approach is look back exercises, which are not representative of the yield sample because of the shift in time, unless archive donor samples are available (6, 16). They also have the major drawback of being limited by recipient susceptibility for HBV infection, death or unavailability and by considerable cost. The clinical significance cannot be addressed by long-term clinical prospective studies but a number of biological examinations can be performed that would provide essential information, including liver biopsies.

An additional issue regarding blood transfusion recipients is the influence of their immune status at the time of contact with HBV. In England for instance over 50% of recipients of blood components have some degree of immunodeficiency that can affect their susceptibility to infection by lowering the threshold of infectivity and the clinical severity of the infection once established. Evidence of this considerable problem has been reported for HBV in severely immunosuppressed recipients of organs (liver) from donors carrying anti-HBc only, anti-HBs and anti-HBc or only HBV DNA (18-20).

The place of occult HBV in the natural history of HBV infection

Occult HBV may have essentially three origins (1):

- Tail end of chronic carriage after years in the non-replicative phase.

- Persistence of low-level replication in the liver and occasionally spilling in blood after recovery from infection characterised by the development of neutralising anti-HBs (21-24).

- Occurrence of escape mutants in vaccinated (breakthrough infection) or unvaccinated individuals that evade detection by current HBsAg capture assays (24, 25).

Although the presence or absence of anti-HBs could be the main marker that distinguishes between chronic and recovered infection and high viral load escape mutants, a majority of NAT yield cases has no detectable anti-HBs because of its relatively short lifespan. As a result, a majority of yield cases presents as "anti-HBc only" with low viral load (<500IU/ml) without the possibility of distinguishing between recovered and chronic infection (2, 3), whereas it cannot be completely excluded that some tail end carriers produce low levels of non-neutralizing anti-HBs. Such distinction is critical since recovered donors are assumed at lower risk of liver disease and infectivity whilst chronic cases are at higher risk of complications and more likely infectious by transfusion.

There are several possible approaches to discriminate between these two forms of occult HBV infection, which include molecular biology, cellular immunology and, to some extent clinical investigation.

Molecular biology

Viral load needs to be determined since it is to a large extent related to infectivity. This can be done with sensitive real time PCR (QPCR) (26) or endpoint titration with more sensitive qualitative NAT systems. Quantitative NAT might not always be reliable with a viral load below 100 copies/ml. While confirmation of a DNA positive result can be provided by the presence of additional serological HBV markers (anti-HBc and/or anti-HBs), the confirmation of samples with DNA as only marker of infection requires special confirmatory strategies. It might be concordance with alternative ultra-sensitive screening assays, repetition of testing, alternative NAT using larger volumes or multiple archived aliquots retained from the plasma bag or

testing look back or prospective samples from the index individual (Lelie supplementary testing of Ultrio reactive samples, 2005). Preliminary observation might indicate a particular infectivity of platelet concentrates possibly related to the preferential binding of HBV to the platelet surface (MP Busch, personal communication).

Sequencing not only in the S gene 'a' region as most people have done to identify mutants that might escape HBsAg capture antibody but also in other areas important for viral replication will be informative (24, 25, 27). One of the question is whether or not there are specific features of occult HBV that explain maintaining such long term low level of replication. To that end, obtaining complete genome sequence would be critical since the pre-S, X, Basic core promoter (BCP) and pre-core regions as well as the polymerase reading frame might be involved. Although not absolute by far, the little data available suggests that recovered infections tend to be of wild type in the pre-core and BCP regions while strains in chronic infections often carry the pre-core 1896 stop codon and the 1762-1764 mutations or deletions of BCP (28).

Preliminary evidence has been provided that the natural history of the infection might be influenced by genotype (10, 29-32). This was well known for genotype B and C but data obtained for genotypes A, D and E seems to also indicate differences in particular in the prevalence of occult HBV (3). In large parts of the world, with standardised and sensitive HBsAg and NAT screening, there is an opportunity to compare the rate of occult HBV infection in previously anti-HBc unscreened donor populations and investigate if there is a relationship with the prevalent genotypes in the donor population. Genotyping of the HBV strains might therefore be important. Furthermore the sensitivity of the amplification system that enables sequencing of a fragment of the S gene is critical for discovering new escape mutations in very low viral load carriers (Koppelman et al, personal communication).

Finally, some discussion has taken place suggesting that viral DNA without HBsAg might originate from incomplete virions or integrated viral DNA released in circulation (33). This issue can be investigated by obtaining evidence of complete genome sequences or capturing viral particles with antibodies to surface antigen and detecting viral DNA (immuno-NAT) or treatment of plasma samples not containing EDTA with DNAse to examine if non particle bound HBV-DNA replication intermediates circulate free in the circulation.

Cellular and humoral immunology

In this situation of near tolerance of the virus by the host, investigating the host immune response is critical. In the frequent situation of anti-HBc as only serologic marker, looking for memory B- and T-cells will be useful. Presumably, recovered individuals will respond to stimulation with both core and surface antigen but chronic infection only with core. Some unexpected specific stimulating epitopes might be discovered (34, 35).

Preliminary data indicates that although anti-HBs is not found in circulation in chronic infection, other antibodies might bind to the virion surface and play a role in limiting infectivity (36). Alternatively, some cases might carry anti-HBs at the virion's surface that need dissociation procedures to be revealed.

The outcome of HBV infection was shown related to genetic determinants, particularly HLA –A, B- or DR- (37, 38). It would be of interest to determine whether occult HBV of one origin or another can be associated with specific alleles, possibly in relation with certain genotypes.

Clinical features

At present, no information is available regarding the potential association of occult HBV with liver disease. Some studies indicate that ALT levels are normal (22, 23). Rare studies examining cases of acute HBV infection in adults that recovered and were re-investigated after one or multiple decades, not only frequently found viral DNA in the liver but also evidence of liver disease (22, 23). Curiously, no histopathological data on the transplanted livers from anti-HBc positive donors that transmitted HBV has been published. However, there might be sufficient clinical evidence to justify performing liver biopsies in selected individuals. If such specimens were obtained, not only evidence of liver disease could be collected but also viral DNA can be quantified and the presence of integrated DNA in hepatocytes be explored. This type of investigation might provide important data regarding the possibility of developing HCC (39, 40).

Results of such investigations should provide the basic knowledge necessary to appropriately counsel donors deferred on the ground of occult HBV.

Objectives of the study

1. To collect samples (component units) from donations carrying HBV DNA after ID NAT or anti-HBc screening but without detectable HBsAg,
2. To characterise these samples molecularly and immunologically
3. To conduct look back studies: identify archived samples from previous donations, trace recipients and collect samples to determine HBV transmission from index donors,
4. To follow up donors and collect samples for molecular confirmation, immunological, genetic and clinical evaluation, including liver biopsy when possible,
5. To fully characterise occult HBV infections taking into account both virus and host parameters and provide the transfusion community with evidence on which to base testing/safety strategies.

Organisation of the study

The study will be conducted under the auspices of the International Society of Blood Transfusion, Transfusion Transmitted infections working party (TTI WP, chair Dr S Wendel). In Seattle on October 15th, 2005 the creation of a subgroup of the WP entitled: '**HBV safety'** was unanimously decided. The subgroup is chaired by Prof JP Allain, University of Cambridge, UK. Other members are: M Busch (Blood Systems), N Lelie (Chiron Europe), H Reesink (Sanquin), M Schmidt (German Red Cross), S Stramer (American Red Cross), M Satake (Japanese Red Cross) and S Wendel (Sirio-Libanes Hospital, San Paolo).

The TTI WP subgroup will be responsible for the generation and communication of data to the ISBT at international or regional congresses. The active arm of the project will be a **collaborative study group** composed of individuals procuring samples, or participating clinically or scientifically to the achievement of the objectives of the study. Three major companies (Abbott Laboratories, Chiron Corporation and Roche) will be part of the study group through one representative and will be instrumental in assembling samples, procuring some of the investigating tools and supporting the study financially. Of course regional collaborations between blood centers and reference laboratories already exist. The outcome of the study group will lead to

major publications. This does not exclude the possibility of collaborating with the ISBT working group to enter the basic data in a central database and to allow special laboratory analyses from surplus archived samples in external laboratories.

Proposed investigations

Investigations will be organised differently whether the origin of samples is HBV NAT or anti-HBc as primary screening test. Where ID HBV NAT is implemented, the initial step will be confirmation of the presence of HBV DNA and depending of the internal logistics in blood centres, additional serologic markers (anti-HBc, anti-HBs titre) will be tested for. All confirmed samples and corresponding donors shall be eligible for entry in the study. Where anti-HBc is the primary screening assay, HBV NAT will be part of the characterisation process of these samples that will include confirmation of anti-HBc, detection and quantification of anti-HBs. Serological markers will then be part of the NAT confirmation process. In addition, anti-HBc reactive donors with low anti-HBs titres (<100 mIU/ml) with or without detectable HBV DNA will be eligible for further study. Anti-HBc positive donors without detectable HBV-DNA with anti-HBs titres above 100 mIU/ml are considered safe for transfusion and will be eligible for re-entry in the blood supply for either labile components or manufacturing of plasma products only, the ultimate decision depending on national or regional regulatory requirements.

The various facets of the investigation described below will be applicable to all samples and donors or recipients considered confirmed positive for HBV DNA.

Collection of Index samples and corresponding data

Plasma and platelet concentrate or plasma separated from red cell when whole blood is used from donors whose screening sample is identified as containing HBV DNA will be kept frozen at –30°C or lower. Such samples will constitute the **Index sample** for any other donor sample retrieved from archive or prospectively collected. Look back recipient samples will be linked to the corresponding archived samples from the donor and, if unavailable, to the Index sample. Part of each sample or the laboratory data will be available to the study group and can be either assembled by representatives of the company manufacturing the NAT screen or stored by each participating blood centre who will notify the study group of the yield case and

arrange for distribution of appropriate samples to the designated laboratories. Published or unpublished donor and recipient data will be made available to the study group and will be entered in the ISBT working group global data base.

Collection of other relevant samples

Blood centres identifying a yield case of occult HBV DNA will make effort to further investigate by searching archived samples from the donor as first step of the look back process and by contacting the donor for prospective collection of samples. The latter are intended to differentiate between window period (WP) yield cases from donors who will subsequently seroconvert to HBsAg and specific antibodies and, for those cases not classified as WP, to exclude a stage of acute resolving HBV infection and confirm the diagnosis of occult HBV in the Index sample in follow-up samples. In addition, whenever possible, the tracing of recipients of previous donation will be initiated and, if successful, recipient samples will be collected.

All samples from the Index donor or linked recipients will be tested for HBV markers including DNA analyses and investigated similarly to the Index sample.

1. *Serological investigations*
   - Anti-HBc should be tested with multiple assays (at least 2). Only samples reactive with two or more assays are considered positive. Titration can be done by limiting dilution or % inhibition of assay read out.
   - Anti-HBs is performed with a quantitative EIA. If the concentration is ≤ 100 mIU/ml, it should be quantified with an assay with a sensitivity below 10 mIU/ml.
   - If possible anti-HBe and IgM anti-HBc are tested in order to exclude the likelihood of early recovery of HBV infection (open core window phase).
   - HBsAg should be re-tested with PRISM if any other EIA or rapid test has been used for screening. If the viral load is ≥500IU/ml, HBsAg should be re-tested with a kit utilizing polyclonal antibodies in order to detect 'a' variants.
   - Dissociation procedures with urea, heat and acidification might be applied to samples classified as 'anti-HBc only' and re-tested with PRISM for the presence of HBsAg, possibly after enrichment of

(immune complexed) HBsAg by ultracentrifugation. When these types of studies are initiated, it is recommended to validate the sensitivity of the system with artificial mixtures of HBsAg and anti-HBs calibrated in IU against international standards.

2. Confirmation and quantification of HBV DNA
Index sample is repeatably reactive with the primary screening NAT. The presence of HBV DNA should be confirmed with at least one of the following options (all assays need a sensitivity ≤ 15IU/ml or 100 copies/ml.
- Chiron HBV DNA discriminatory
- Roche HBV COBAS Ampliscreen with 1 ml sample preparation
- Nested PCR in house
- Sensitive qualitative QPCR
- Special assays with maximum sensitivity developed by the NAT manufacturers for research purposes or development of future screening platforms (GenProbe or Roche).

3. Quantification of viral load and estimation of the amount of transfused virus
Quantification can be done by real time PCR against a calibrated internationally used standard (WHO/NIBSC or alternative calibrated internationally applied real virus QC standards such as PeliCheck, ISS, PEI or JRC standards) using primers and probes verified as detecting with equal sensitivity (≤15 or 100 mIU/ml) all HBV genotypes. Since quantification of a large proportion of occult HBV carriers with concentrations at or below the 95% detection limits of the current screening systems will not be reliable, it is possible to estimate the viral load by testing two-fold dilutions in optimised qualitative NAT systems, either ultra-sensitive research assays in development by the NAT manufacturers. NAT systems used by reference labs employing ultracentrifugation of the samples for which the assays have been calibrated with un-manipulated whole particle viral standards calibrated in genome equivalents might also be useful. Calibration in genomes in addition to calibration in IU is relevant to relate the infectivity status of the donation to the amount of infused virus by the blood component. In window phase donations 10 (1-100) genomes may be infectious. Both for window phase and occult carriers it is important to estimate the amount of infused Dane particles

from the donation and to gain from the look back studies information about the relative infectivity of HBV in different types of HBV-DNA positive, HBsAg negative donations.

4. *Molecular biology*

    a. Sequencing

    Sequencing will be used to answer several questions:

- Genotype
- Variation of 'a' epitopes of the S gene
- Identification of known mutations or deletions/insertions in the BCP, pre-core, Pre-S1 and pre-S2 regions
- Identification of new genetic differences potentially involved for decreased viral replication or escape from HBsAg detection
- Demonstration of origin of HBV infection between donor and recipient strains in paired samples
- Phylogenetic analysis
- Intra-strain variability (quasispecies) or mixed genotype infection
- Inter-genotype recombination

All these answers can be obtained by full genome sequencing of multiple clones. However, this approach is highly labor intensive, expensive and limited in sensitivity to ≥ 25 IU/ml. Short of this, options are:

- Full genome consensus sequence
- BCP-Pre-core nested amplicon (0.05-1Kb
- Pre-S-S region 1.2Kb
- BCP region 0.3 Kb
- Pre-core
- S region containing 'a' region
- Pre-S1-pre-S2

To assess intra-strain variability and genotyping, the pre-S/S region is probably the most informative.

It is recommended to use ultracentrifugation of at least 1 ml of serum/ plasma for enrichment of HBV prior to amplification of the fragment to be sequenced. Before applying the PCR method it is recommended to validate the sensitivity with diluted

un-manipulated whole HBV standards (e.g. PeliCheck) to ensure that levels below 30-100 geq/ml can be detected.

b. Infectivity in vitro

Some HBV strains might be suspected of carrying genomic changes responsible for decreased replication. These strains can be used for infectivity in permissive cell lines. Similar changes can be engineered in known strains and the effects compared to the wild type.

c. Differentiation between encapsidated vs non-encapsidated or incomplete HBV genomic material

This issue can be investigated by several means:

- obtaining complete clone genome sequence suggest full virus
- viral particles can be captured with immobilised anti-HBs and DNA detected
- CPD plasma unit-derived material (not containing EDTA) can be treated with DNAse and if, when compared to a whole virus standard of similar viral load, a significant reduction of the HBV load may indicate the presence of naked DNA .

This identification of full virions is critical to correlate with infectivity.

5. Donor immunology

In the context of differentiating between occult HBV originating from recovered or chronic infection, information can be obtained from both humoral and cellular immunity.

Humoral, Antibody to HBs detection, including complexed to virus was mentioned in the serology section. Other investigations can be conducted such as the detection of memory B-cell detected by ELISpot type of assay or flow cytometry. Anti-HBs can also be detected in serum following a single boosting injection HBV recombinant vaccine of the S region. Monitoring anti-HBs 6-12 days post-injection can detect a secondary response in recovered individuals with recurrent detectable HBV-DNA having lost detectable anti-HBs.

Cellular. PBMC can be prepared from investigated donors in the course of follow-up. PBMCs can be stimulated with either recombinant proteins or peptides to identify the presence of committed memory T-cells. An ELISPot system for

proliferative response alone or in conjunction with detection of cytotoxic response can be used.

In parallel with the cellular response HLA-A, HLA-B and HLA-DR genotyping can be applied to the collected PBMC in order to investigate the potential association of occult HBV and genetic markers.

6. Additional clinical studies

The clinical aspects of occult hepatitis B have not been well studied for lack of sufficient number or representative cases. Either they came from hepatology and presented with clinical symptoms or from occasional discoveries. There is therefore an opportunity to draw the bid picture.

a. Donor

As much as possible, classical liver disease investigations should be conducted such as:

- History (jaundice, acute hepatitis, risk behaviour, family cases etc)
- Testing for ALT, AST, fibrosis markers and markers of inflammation
- Imaging of the liver whenever possible
- Whenever possible and ethically acceptable, liver biopsy should be performed. While it might be ethically difficult without evidence of liver disease, when HBV DNA is associated with elevated ALT level or any other evidence of liver disease is present, it becomes legitimate.

b. Recipients

Clinical information regarding recipients is to be collected:

- Underlying disease
- Treatment received in particular drugs that might be immunosuppressive
- Searching for any pre-transfusion sample

Implementation of plan and distribution of tasks

1. Blood Centres

It would be the responsibility of the individual centres to undertake HBV DNA confirmatory (chapter 2) as well as confirmation of anti-HBc. Some centres might also want to conduct some of the testing for serologic HBV markers listed in chapter 1 above.

It would also be the responsibility of individual Blood Centres to undertake the retrieval of archived samples and initiate the look back. Recalling the donors for information, counseling and collection of prospective samples will be done by the Centres.

Blood Centres might refer the deferred donors with occult HBV to local hepatologists for clinical investigation as described in chapter 6.

2. Special investigations

It would be highly desirable that specialised investigations such as described in chapters 2-5 be conducted centrally by a limited number of laboratories with experience in each particular areas.

The molecular biology part of the study can be divided according to subchapters or geographical areas.

Although this is not optimal, the cellular immunology part can be done with cells frozen in liquid nitrogen in a single location or several if required by geography.

For practical purposes, the HBV safety study group will be subdivided in two subgroups: one for areas where anti-HBc is the initial screening such as North America, northern Europe, Australia and Japan and a second for areas where the primary screening will be HBV NAT preferably in ID but also in pools of 10 or less plasmas such as Southern and Eastern Europe, South East Asia, the Middle East and sub-Saharan Africa. For each of these two branches, separate reference laboratories will be identified.

Dissemination of information

The formation of the study group shall be announced by the chairman of the TTI WP within ISBT. Anticipating a situation that data collected by various regional investigators will be published, an attempt will be made to present preliminary data of a global meta-analysis at the international ISBT congress in Cape Town, either within the WP or at a suitable session within the congress if sufficient data is collected in time. It is proposed to use the sample repository currently established in South-Africa and Spain (virgin ID-NAT countries) and the Netherlands and Germany (virgin anti-HBc countries) as the starting point of this study. It is hoped that available data from other countries will be entered in the ISBT database.

Ultimately, the data of the analyses will be disseminated through peer-reviewed journals after approval by the WP of manuscripts submitted by the chair of the study group to the HBV safety subgroup.

Impact of data collected

It is anticipated that the data collected by the study group will provide evidence on which health authorities can base specific strategies to address HBV blood safety. This information will be comprehensive including testing, epidemiology, efficacy of screening, risk of transmission by transfusion and clinical significance. This ISBT initiative attempting to standardise investigation and collect global blood screening yield data in a central database is a pre-requisite to understand the value of the laboratory parameters for the infectivity of blood donations. Ultimately this will be a unique tool for regulators to establish meaningful requirements to ensure the safety of the blood supply.

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