RESEARCH NOTE

INSUFFICIENT EVIDENCE OF TT-VIRAL ETIOLOGY OF POST HEPATITIS APLASTIC ANEMIA

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Abstract. Post-hepatitis aplastic anemia is a rather rare pathologic condition of as yet unclear etiology especially as hepatitis viruses A to G have been excluded as the potential agents responsible. The novel TT virus, a single-stranded DNA virus first isolated from the serum of a patient with post-transfusion hepatitis in Japan might cause this condition. Therefore, our group subjected the sera of two children with post-hepatitis aplastic anemia to semi-nested PCR using primers specific for detection of TTV DNA. Although TTV DNA was not detectable in either sample it might be speculated that, like hepatitis viruses A to G, TTV could be found associated with this condition whereas it certainly does not constitute its sole etiologic agent.

Post-hepatitis aplastic anemia constitutes a rather rare pathologic condition the etiology of which to date has eluded elucidation. During the hepatitis phase, the clinical symptoms resemble those encountered in acute hepatitis caused by either viral infection or toxins. Most cases of aplastic anemia have been investigated for their association, at first, with hepatitis A or hepatitis B virus, HAV and HBV, respectively (Zeldis *et al*, 1983; Sandberg *et al*, 1984; Liang *et al*, 1990), neither one of which could be established as the causative agent. Moreover, with neither one of the newly discovered hepatitis viruses C to G a direct link to the etiology of aplastic anemia could be found (Hibbs *et al*, 1992; Poovorawan *et al*, 1997).

Hepatitis T virus (TTV) is a novel hepatitis virus first isolated by means of representational difference analysis (RDA) as a clone of 500 nucleotides from the serum of a patient with post-transfusion hepatitis of unknown etiology exhibiting characteristically elevated ALT levels (Nishizawa *et al*, 1997). Subsequently, this virus has been molecularly cloned and characterized by the same group of researchers as a non-enveloped, single-stranded DNA virus of which approximately 3.7 kb harboring two potential open reading frames have been sequenced. Upon sequencing the 3.7 kb clone and subjecting it to homology search, no nucleotide sequences showing a significantly high sequence homology to it could be detected (Okamoto *et al*, 1998).

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To date, five different genotypes of TTV have been isolated from serum of infected individuals in Japan where the virus has been shown to exhibit a high prevalence in patients at risk for parenteral exposure, such as hemophilia and hemodialysis patients, or intravenous drug users (IVDU). Likewise, TTV was detected among patients with non-A-to-G fulminant hepatitis and chronic liver disease at a frequency amounting to almost 50 % (Okamoto *et al*, 1998).

The present study was aimed at determining the possible role of TTV as the causative agent of post-hepatitis aplastic anemia exemplified by two pediatric cases extensively investigated for associations with hepatitis viruses A to G, Epstein-Barr virus (EBV), and cytomegalovirus (CMV), none of which could unequivocally be termed responsible for this affliction. The clinical details of these two pediatric patients have been reported elsewhere (Poovorawan *et al*, 1997). The patients developed aplastic anemia 4 and 2 months after the onset of symptomatic hepatitis, respectively.

The investigation for the presence of TTV DNA in the sera obtained from both patients during the acute phase of hepatitis and in the convalescence phase was performed by semi-nested PCR according to the procedure described below.

Hepatitis TT virus DNA detection

DNA extraction: DNA was extracted from 50 µl of serum, twice per each sample, with proteinase K/SDS in Tris buffer, followed by phenol/chloroform extraction and ethanol precipitation. The pellet was dissolved in 20 µl of sterile water and directly sub-

jected to the polymerase chain reaction.

TTV-DNA detection: TTV-DNA was detected by polymerase chain reaction using semi-nested primers. The amplification reaction was performed in a 50 µl reaction volume containing 1 U of Taq polymerase (Perkin Elmer Cetus), and each of four deoxynucleotide triphosphates at a concentration of 200 µM, primer pairs NG 059 and NG 063 for the first round, and NG061 and NG 063 for the second round, respectively, at a concentration of 1 µM each, 10 mM Tris, 1.5 mM $MgCl_2$ and 5 µl of each DNA sample. According to Okamoto *et al*, (1998) the nucleotide sequences of the TTV primers derived from the N-22 region were: NG 059 (5′ CAG ACA GAG GAG AAG GCA ACA TG 3′), NG 061 (5′ GGC AAC ATG TTA TGG ATA GAC TGG 3′) and NG 063 (5′ CTG GCA TTT TAC CAT TTC CAA AGT T 3′). The first round amplification reaction using primer pair NG 059 and NG 063 was performed for 30 cycles (denaturation at 94°C for 36 seconds, annealing at 55°C for 42 seconds, and extension at 72°C for 1.30 minutes, hold 72°C for 10 minutes). The second round of amplification was performed using 2 µl of the PCR product along with primer pair NG 061 and NG 063 for 30 cycles under identical conditions. Upon conclusion of the PCR the reaction mixture was spun for 1 minute at 10,000 rpm, and 10 µl each of the amplified DNA were fractionated by electrophoresis in a 2% agarose gel stained with ethidium bromide and visualized under UV light. The product band will show at 271 base pairs. The gels were photographed on a UV light box. Sera obtained from IVDU and known to be positive for TTV-DNA and sterile water were used as positive and negative controls, respectively.

The results thus obtained showed that both cases of post-hepatitis aplastic anemia previously proven negative for hepatitis viruses A to G, as well as for EBV and CMV (Poovorawan *et al*, 1997) were equally negative for the novel post-transfusion hepatitis virus TT.

Several investigators have tried to determine the etiology of the particular hepatitis preceding post hepatitis aplastic anemia based on the hypothesis that some agents might affect both the liver and stem cells of the bone marrow and thus result in aplastic anemia. Since neither of the known hepatitis viruses A to G could unequivocally be termed responsible the possibility of another as yet unknown virus being the cause ought to be investigated. After the Japanese team has demonstrated that the new TT viruses are parenterally transmitted, the present study was focused on the role of TT virus in the course of the underlying hepatitis. The two patients reported here had no history of blood transfusion and were healthy children before developing the disease. As previously reported (Poovorawan *et al*, 1997), we had examined them already for hepatitis viruses A to G and found them negative for those agents, as they also turned out now for the novel TT virus. Hence, as with hepatitis viruses A to G, although TTV may be associated with aplastic anemia, it does not appear to be its sole causative agent. Yet even so, discounting viruses not directly implicated in chronic liver disease might prove shortsighted on the long run. The time elapsed between the discovery of parvovirus B19 (Cossart *et al*, 1975). and recognition of its connection with aplastic anemia (Pattison *et al*, 1981) may serve as a typical example.

To date, five different genotypes of TTV have been characterized by the Japanese group, which, as we are faced with a novel virus, might well represent the top of the iceberg. Hence, although we have selected our set of primers according to the conserved region found in the 5 genotypes classified by (Okamoto *et al*, 1998), it is questionable if all the genotypes present in Thailand can thus be detected, especially since our data as to the prevalence of TTV among members of high risk groups have shown a lower prevalence than that observed in Japan. For example, our group detected TTV DNA in 33 % of IVDU (Poovorawan *et al*, 1998), whereas the group from Japan reported 40 % positive by semi-nested PCR (Okamoto *et al*, 1998).

In conclusion, the etiology of post hepatitis aplastic anemia still remains enigmatic. Various researchers, as well as our group, have investigated patients with this severe disease for a causal link to hepatitis viruses A to G, now also including the novel hepatitis TT virus. Yet, to date only associations with hepatitis viruses C and G, most probably due to multiple blood transfusions, could be ascertained, leaving the question regarding the causative agent open to further investigation.

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