Variants within the "a" Determinant of HBs Gene in Children and Adolescents with and without Hepatitis B Vaccination as Part of Thailand’s Expanded Program on Immunization (EPI)

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Theamboonlers, A., Chongsrisawat, V., Jantaradsamee, P. and Poovorawan, Y. Variants within the "a" Determinant of HBs Gene in Children and Adolescents with and without Hepatitis B Vaccination as Part of Thailand's Expanded Program on Immunization (EPI). Tohoku J. Exp. Med., 2001, 193(3), 197–205 — A total of 2229 children selected from five distinct areas of Thailand were screened for HBs antigen (HBsAg) by ELISA. Out of 51, forty-nine HBsAg-positive children were further examined for HBV-DNA by the polymerase chain reaction, utilizing the region of the hepatitis B Virus (HBV) genome encoding the major antigenic epitopes of hepatitis B surface antigen. Direct automated sequencing of the "a" determinant region revealed 11 of 49 children to display variable mutations. The vaccinated and nonvaccinated children had amino acid variants clustered between residues 120 and 160. Mutations between residues 120 and 160 were found at higher frequency in the vaccinated group (4/13; 30.8%) than in the nonvaccinated group (7/36; 19.4%), but this was not statistically significant. Infections with new HBV variants are contracted either vertically or horizontally within the group having received the vaccine, a finding confirmed by the presence of amino acid substitutions critical for immune escape. Hence, neither vaccine nor IgG has any apparent effect on those variants and the children turn into HBV carriers. However, the current vaccination program still efficiently protects perinatal transmission of HBV and unless long term studies lead us to conclude otherwise, inclusion of the variant strain(s) into a new vaccine formulation is not deemed necessary. —— HBsAg; HBV mutants; hepatitis B vaccine; "a" determinant

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Prevention of viral infections involves the development of vaccines aimed at targeting the respective viral envelope; in particular those coat proteins that serve as the epitope first recognized by the host’s humoral and cellular immune response. Recently, novel viral variants have caused increasing concern in that due to mutations in their envelope proteins they may escape the vaccines currently in use (Carman 1994).

In the course of part of its replication hepatitis B virus (HBV) employs reverse transcriptase which contrasting DNA polymerase lacks proofreading capacity and hence, allows for a higher number of mutations to occur than is commonly observed among DNA viruses (Summers and Measons 1982). Some of the thus resulting variants may prove advantageous in that for example, they harbor greater replication potential and thus become dominant. Thereupon, those so-called escape mutants are positively selected by the host’s humoral and cellular immune response from the pool of variants with similar replication potential (Prange et al. 1995).

Three genes which are translated from distinct mRNAs (Tiollais et al. 1981) and collectively known as hepatitis B surface antigens (HBsAg) have been found to code for the envelope protein of HBV. The dominant epitope cluster of HBsAg lies within its major hydrophilic region (MHR) termed the “a” determinant. Alteration in this region can affect B-cell epitopes of HBsAg engendering immunological escape from the host’s defenses elicited by either vaccination or previous infection (Water et al. 1987). The “a” antigenic determinant of HBsAg is located between amino acids (aa) 124 and 147 (Howard and Allison 1995) with the antibody to HBsAg binding to aa 139–147. Thus, the nine amino acids in this region particularly affect the binding of antibody to HBsAg, which comprises 226 amino acids encoded by the S gene.

In numerous areas worldwide HBs escape mutants have been reported to exhibit a change of glycine (Gly) to arginine (Arg) at position 145 as the mutation commonly observed. This particular change has been reported to induce vaccine escape mutants along with nucleotide changes resulting in amino acid substitution elsewhere in the “a” determinant.

A change in the molecular environment, for example by administration of vaccines, monoclonal or polyclonal antibodies might exert selective pressure and hence, cause the emergence and eventual dominance of minor strains. Various examples of escape variants are caused by point mutations in the “a” determinant (Neurath et al. 1990). Generally, point mutations not only occurring in the key residues for subtype determinants but also in positions within the common “a” determinant have been shown to lead to other variants which apparently lack subtype expression (Carman et al. 1990; Magnus and Norder 1995) suggesting the “a” determinant to possess a highly variable conformational structure. There have been numerous reports on mutation within the “a” determinant detected among HBV carriers displaying anti HBs (Yamamoto et al. 1994), liver transplant patients receiving HBlg therapy (Carman et al. 1990; Okamoto et al. 1992) and failure of immunophylaxis in neonates (Oon et al. 1995; Poovorawan et al. 1998).

The EPI program has been introduced in Thai in 1989 and various studies have shown that the vaccine can prevent HBV infection at a rate exceeding 80% (Chunsuttiwat et al. 1997; Poovorawan et al. 2000a). However, a small percentage of babies are still found to be infected with HBV. This failure of protection after immunization has reminded unclear; it may be due to infection already established before immunization, or due to HBV having undergone a mutation in its “a” antigenic determi-
nant, rendering the vaccine useless. Subsequent to these mutations monoclonal antibody binding is almost totally, polyclonal antibody binding occasionally abrogated (Water et al. 1987).

In the present study, we performed serological screening in five areas representing the North, South, Northeast, and Central parts of Thailand, we then applied PCR and direct sequencing on the vaccinated and nonvaccinated groups in these areas to establish the respective mutation types in the "a" antigenic determinant and their distribution in Thailand. An obvious correlation between the antigenic and molecular characteristics of the HBV isolates examined has become apparent in this study.

**Materials and Methods**

**Population study**

The locations for blood sample collection comprised five provinces representing geographically distinct areas in Thailand. Based on the outcome of a previous study (Poovorawan et al. 2000a). There were 51 children and adolescents, aged between 6 months and 18 years, with HBV carriers. We used 49 samples (two sera were not available) serologically positive for HBsAg (Abbott Laboratories, North Chicago, IL, USA) in both the vaccinated (complete and incomplete) and the nonvaccinated group. Nine out of 49 had received the complete HBV vaccination course, four incomplete vaccinations cause (however they received the first dose of vaccine at birth), and the remaining 36 had not received any HBV vaccination.

**Determination of Amino acid mutations**

**HBV DNA extraction**

DNA was extracted in duplicate from 50 µl serum, each with proteinase-K/SDS in Tris buffer, followed by phenol/chloroform extraction and ethanol precipitation. The pellet was dissolved in 20 µl sterile water and directly subjected to nested PCR-based amplification.

**HBV DNA detection**

The selection of the primer pairs for HBV DNA amplification was based on sequence data provided by Dr. M. Yano at the WHO Collaboration Centre, Nagasaki, Japan. HBV DNA was amplified by nested PCR in an automated thermocycler 9600 (Perkin Elmer Cetus, Branchburg, NJ, USA) as described elsewhere (Theamboonlers et al. 1999).

**DNA purification and sequencing**

The PCR product was purified for sequencing using the QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's specifications and subsequently subjected to 2% agarose gel electrophoresis in order to ascertain its purity.

For determining the concentration of the amplified DNA, we measured the absorption at 260 nm of every sample in an UV spectrophotometer (Shimadzu UV-160 A, Tokyo). The concentration was calculated according to the formula 1 OD 260 = 50 µg double-stranded DNA. Between 10 and 30 ng/µl (3–6 µl) of each DNA sample were subjected to cycle sequencing using dye-labeled terminators (8 µl) and 3.2 pmole of specific primer in a final reaction volume of 20 µl by ABI Prism 310 Genetic Analyser (Perkin Elmer Cetus). This round of amplifications was performed according to the manufacturer's specifications, using primer S2-1 to amplify the particular DNA strand of interest for further sequencing (Theamboonlers et al. 1999). The extension products were subsequently purified from excess unincorporated dye terminators by ethanol precipitation according to the manufacturer's specifications (Perkin Elmer Cetus) and subjected to sequence analysis by the ABI Prism 310 Genetic Analyser.
For all subsequent steps we referred to the ABI Prism 310 Genetic Analyser user's manual (Perkin Elmer Cetus).

**Sequence analysis**

The nucleotide sequences obtained were then translated into the corresponding amino acid sequences, based on the universally valid triplet code applying the Navigator software. The results were compared with the amino acid residues specifying determinants of HBsAg (Ono et al. 1983; Norder et al. 1992), with particular emphasis on HBV S-protein variations between amino acids 120-160 related to serological subtypes.

The subtypes, genotypes and amino acid mutations were determined according to the classification described elsewhere (Magnius and Norder 1995; Norder et al. 1992)

**RESULTS**

Of the 2229 subjects selected from five provinces, 49 of those 51 who were HBsAg positive by ELISA (Abbott Laboratories, North Chicago, IL, USA) yielded a band of the predicted size (249 bp) on agarose gel after nested PCR using primer pairs chosen for amplification of the "a" determinant of the S gene. Those 49 samples showed 46 of adr and 3 of adw subtype as shown in Table 1.

<table>
<thead>
<tr>
<th>Province</th>
<th>No.</th>
<th>Genotype</th>
<th>Subtype</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>B1 A C</td>
<td>adr Adw</td>
</tr>
<tr>
<td>Lampang</td>
<td>16</td>
<td>1 1 14</td>
<td>14 2</td>
</tr>
<tr>
<td>Lopburi</td>
<td>12</td>
<td>0 0 12</td>
<td>0 12</td>
</tr>
<tr>
<td>Udon Thani</td>
<td>8</td>
<td>0 0 8</td>
<td>0 8</td>
</tr>
<tr>
<td>Chon Buri</td>
<td>12</td>
<td>1 0 11</td>
<td>1 0 11</td>
</tr>
<tr>
<td>Nakornnristharmraj</td>
<td>1</td>
<td>0 0 1</td>
<td>1 0</td>
</tr>
<tr>
<td>Total</td>
<td>49</td>
<td>2 1 46</td>
<td>46 3</td>
</tr>
</tbody>
</table>

Subsequent sequencing revealed the distribution of HBV genotypes, subtypes and variation of the "a" determinant gene mutations among the children studied. Eleven out of 49 sequences were found to have mutations, as shown in Table 2. Of these, 4/13 (30\%) vaccinated children had the sporadic mutations M133T, S143W, D144A and C149W. As for nonvaccinated children, 7/36 (19\%) sequences showed the mutants C139W, S143L, G145R, C149G/W, S154K/E and S155F. There were no statistically significant different between the vaccinated and nonvaccinated groups.

**DISCUSSION**

In this study, 2229 children selected from 5 geographically distinct areas of Thailand were screened for HBsAg using ELISA. We found 51 (2.3\%) of these HBV positive by ELISA, and since both vaccinated and nonvaccinated groups were included, maternal infection and vaccine escape mutants are presumed to have occurred. Sequencing 11 of the 49 (2 sera were not available) children’s PCR products (22.4\%) revealed variable mutations in the "a" determinant. Despite immunoprophylaxis, some were infected with HBV displaying amino acid changes within the "a" determinant. Thirty-eight of the 49 (77.6\%) samples indicate that the children have been vertically infected despite standard immunoprophylaxis, possibly with the wild type strain.

The specific sequence on the "a" determinant has been shown to code for the antigenic epitope of the virus and hence is most prone to environmental pressure, resulting in mutations (Hsu et al. 1999). Our data on vaccinated children had the mutations M133T, S143W, D144A and C149W and nonvaccinated children showed the mutants C139W, S143L, G145R, C149G/W, S154K/E and S155F. It was found that T126A, Q129H and M133L mutation occurred in infants with chronic infections despite immunoprophylaxis (Oon et al. 1995). Of
<table>
<thead>
<tr>
<th>HB vaccination at birth</th>
<th>No.</th>
<th>Amino acid sequence of “a” determinant</th>
<th>Genotype</th>
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<tr>
<td>Yes</td>
<td>9</td>
<td>PCKTCTIPAQGTSMFSPCCCTKPSDGNCTCIPISWAFAR</td>
<td>C</td>
</tr>
<tr>
<td></td>
<td>2</td>
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<td>C</td>
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<td>T</td>
<td>C</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>WA</td>
<td>C</td>
</tr>
<tr>
<td>No</td>
<td>1</td>
<td>PCKTCTIPAQGNMSMFSPCCCTKPTDGNCTCIPISWAFAK</td>
<td>A</td>
</tr>
<tr>
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<td>2</td>
<td>PCKTCTIPAQGTSMFSPCCCTKPTDGNCTCIPISWAFAK</td>
<td>B1</td>
</tr>
<tr>
<td></td>
<td>26</td>
<td>PCKTCTIPAQGTSMFSPCCCTKPSDGNCTCIPISWAFAR</td>
<td>C</td>
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<tr>
<td></td>
<td>2</td>
<td></td>
<td>C</td>
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those, the T126A mutation impacts the \( w \) subtype determination and thus affects the recognition properties of the surface antigen (Magnius et al. 1995). With the P127T, mutant residue 127 is the critical site for the serological variations between \( w1/w2 \) (Magnius et al. 1995); F134L is an HBV mutant recovered from one vaccinated Taiwanese carrier child (Lee et al. 1997); C149R represents a variant in Sardinia (Carman 1997), where position 149 has previously been shown to be important for its immunological activity and D144A and G145R which have been proposed to be vaccine-induced escape mutants (Carman et al. 1990; Harrison et al. 1994).

The novel mutations of T126S, T143M, S143W, D144H, and T143L have been seen in sporadic isolates from Spain (Wallace et al. 1994). The nine new variants were T125A, C138S, T140R/L/I/P, N146S, C147R, T148I, and W156L, also seen in Taiwan (Hsu et al. 1999). G145R is the common mutation in the world. In this study we also found novel variants in either the same position or different positions, which can be classified as specific mutants of the “\( a \)” determinant in Thailand.

In 11 children with detectable mutants, the mean age was lower in the vaccinated compared with the nonvaccinated group. The emergence of the mutants might have been triggered as a consequence of the universal vaccination program. The mutation can be found both in natural isolates and those mutants, selected over a short period subsequent to immune therapy, immunization prophylaxis with HBIG, or natural anti-HBs seroconversion (Carman et al. 1997). In this study we found new mutation variants in the “\( a \)” determinant within the vaccinated group which supports the hypothesis, consistent with other studies (Yamamoto et al. 1994; Oon et al. 1995), that there are many subtype related variants occurring after active immunization.

The stretch of amino acids 140-149 displays the greatest local hydrophilicity (Hopp and Wand 1981), containing predominantly antigenic “\( a \)” determinants, and constituting the major neutralizing epitope; positions 125-129 constitute the subtype specificity determinant (Ono et al. 1983; Magnius et al. 1995). In this study, we found mutations both in vaccinated and nonvaccinated samples, suggesting that natural human immunity and vaccination could induce selection of both variant types. The mutation at the neutralizing epitopes may result in larger antigenic changes (Hopp and Wand 1981). The novel neutralizing antigen related mutants might imply vaccine-selected mutations to more likely occur at the neutralizing epitopes in the subtype specificity determinant. This causes larger immunological changes, as has been suggested by a previous study of vaccine-induced mutations occurring at residues 139-147 (Howard and Allison 1995; Carman et al. 1997).

The G145R mutant is well known and can be found in many areas worldwide, also position 126 has been shown to exhibit various degrees of altered binding of HBsAg to several monoclonal antibodies (Chiou et al. 1997). The mutations of residues within the “\( a \)” determinant (T126A, M133L, F134L and T143M) were found to alter the epitope’s hydrophilicity and conformation, causing changes in the \( \beta \) turn secondary structure (Zhang et al. 1996). The substitution of amino acid residues within the presumed second loop (139-147) of the “\( a \)” determinant may involve alterations within the side chain of amino acid residues, i.e., a loss of a negative charge in the D144A mutant. Substitutions at positions 147 or 149 also cause the loss of antigenicity (Mangold et al. 1993). Based on this information we can identify many mutants that are capable of escaping HBV vaccine prophylaxis.

Amino acids 124-147 of the S gene product, consisting of two loop structures maintained by a disulfide bond constitute the B-cell epitope of HBsAg. Genotypes with this mutation can escape the immunological defenses induced by
vaccination or by therapeutically administered monoclonal anti-HBs, and remain in circulation (Harrison et al. 1991; Okamoto et al. 1992; Yamamoto et al. 1994).

Escape mutants within the S gene pose a risk to the community because neither the vaccine nor HBIG are effective in preventing HBV infection. Also, blood transfusions can cause transmission of these variants.

The “a” determinant of the S-gene product, located between amino acid positions 120 and 160, has been recognized as the sequence crucial for antigenic epitope structure and conformation and hence, for the immune response. The vaccine specifically designed for the mutant variant, is not available the current vaccination programs are effective in protecting from perinatal transmission of wild type HBV, including under the field condition (Poovorawan et al. 2000b), which comprises the majority of circulating HBV in Thailand. We found an increase in mutated residues and more mutations at neutralizing epitopes within the “a” determinant of HBsAg in vaccinated children. These became carriers because of a selective advantage in the presence of anti-HBs. The protective efficacy of two licensed hepatitis B vaccines has been evaluated in chimpanzees by challenge with the mutant virus strain G145R, displaying arginine-substituting glycine at the surface gene codon 145. The chimpanzees thus challenged did not develop evidence of HBV infection compared with the controls (Ogata et al. 1994). However long-term study on the impact of mutant strains need further studies.

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