Does apolipoprotein E polymorphism influence susceptibility to malaria?

M A Wozniak, E B Faragher, J A Todd, K A Koram, E M Riley, R F Itzhaki

Outcome of infection varies greatly among people, and in the case of three very different viruses, it is determined by apolipoprotein E (apoE) genotype. apoE might affect outcome of malaria infection also, since apoE protein and the protozoon (like the viruses) share cell entry mediators (heparan sulphate proteoglycans and/or specific apoE receptors). apoE polymorphisms give rise to protein variants that differ in binding strength to these mediators; thus, the extent of competition between apoE and protozoon for cell entry, and hence magnitude of protozoon damage, might depend on apoE isofom. Genotypes of infants infected with malaria were examined. It was found that apoE e2 homozygotes became infected at an earlier age than those carrying the other genotypes, the difference being statistically significant. Parasite densities, all of which were low, did not differ significantly. This effect, although based on small numbers, suggests that apoE e2 may be a risk factor for early infection.

Malaria affects about 200-300 million people at any one time and more than one million, mostly children, die as a result each year. The age at which primary infection occurs varies greatly; some infants are infected at or very soon after birth whereas others become infected later. This depends in part on the intensity of malaria transmission but genetically determined differences between people may also influence risk of infection. Numerous genetic factors have been shown to influence the outcome of malaria infection, but none has been shown to affect the risk of infection per se. If a genetic factor involved in susceptibility to infection, or in severity of response to infection, were to be identified, this would contribute to our understanding of the mode of infection and pathogenesis of the disease and might contribute to the development of preventive measures to protect those who are most at risk.

One possible host factor that might affect susceptibility to infection is apolipoprotein E (apoE for gene, apoE for protein). There are three main apoE alleles (apoE e2, e3, and e4) encoding three main isoforms (apoE2, 3, and 4), which differ in primary structure at two sites, apoE3 having cysteine at residue 112 and arginine at 158, apoE4 having arginine and apoE2 having cysteine at both sites. ApoE3 transport lipids in the blood and are involved in repair of tissue damage.

Several studies have shown that apoE determines the extent of damage in the case of certain diseases caused by viruses. Firstly, apoE governs the outcome of infection with herpes simplex virus type 1 (HSV1); apoE e4 and apoE e2 are risk factors for herpes labialis and herpes simplex encephalitis, respectively. Moreover, possession of an apoE e4 allele and presence of HSV1 in brain is a strong risk factor for Alzheimer’s disease. Secondly, carriage of apoE e4 is a risk factor for dementia and peripheral neuropathy in pre-AIDS HIV infected subjects, and, finally, apoE e4 is strongly protective against severe liver damage caused by hepatitis C virus (HCV).

One of the possible mechanisms of the virus-apoE interactions might relate to viral entry. ApoE enters cells via members of the low density lipoprotein receptor (LDLR) family and/or heparan sulphate proteoglycans (HSPG) on the cell surface. The viruses HSV-1, HIV, and HCV also use one or other of these entry mediators. Differences in apoE isofom binding to the receptors (which are cell type dependent) have been noted, and so the extent of viral spread and hence of damage might well depend on the specific isoform(s) of the host (as well as on the tissue involved). The malaria sporozoite (the form in which the protozoon invades hepatocytes in the liver, the initial target organ) also uses a member of the LDLR family as well as HSPG to enter hepatocytes, suggesting that apoE might also influence the outcome of infection with this parasite. Further, a recent study has shown that a small heparin oligosaccharide, one which binds specifically to apoE, blocks interaction of the malarial circumsporozoite protein with human hepatoma (HepG2) cells in culture. The extent of sporozoite entry into liver and/or its spread in that organ could be affected by the apoE isofom of the host, since the affinities for the isofoms for receptors on HepG2 cells in culture differ greatly, that of apoE4 being much greater than that of apoE2 and apoE3. Thus, in the case of apoE4 carriers, there could be less sporozoite entry than in those carrying the other alleles.

In the present study, we have examined apoE genotypes of a birth cohort of Ghanaian infants, being raised in an area of stable, perennially endemic Plasmodium falciparum malaria transmission, to find if there is any association with their risk of becoming infected with malaria at different ages. We have sought also the possibility of an association of apoE genotype with parasite density in blood. Overall, mean time to infection differed significantly between genotypes and was significantly shorter in apoE e2 homozygotes, indicating that apoE genotype affects risk of malaria infection. Interestingly, the frequency of the apoE e2 allele in the Ghanaian population was unusually high, perhaps suggesting that this allele is under positive selection. However, these findings must be interpreted with caution as they are based on a small number of affected infants.

METHODS
Study area and cohort
The study was conducted in Prampram (population 8000), approximately 50 km east of Accra on the south coast of Ghana, in an area of coastal savanna. Malaria transmission in the area is perennial (although with some seasonal variation), stable, and of low to moderate intensity (5-10 infectious bites per person per year). P. falciparum accounts for 98% of all detected infections. Informed consent was obtained from all volunteers and ethical approval for all procedures was obtained from the ethical review committee of the London
School of Hygiene and Tropical Medicine, the institutional review board of the Noguchi Memorial Institute of Medical Research, University of Ghana, and the Ghanaian Ministry of Health. After giving informed consent, a consecutive sample of 110 mothers and their newborn infants was recruited into the study. A heparinised heel prick blood sample was obtained from each child on the day of delivery, at 2, 4, and 6 weeks of age, and then every four weeks. The child’s axillary temperature was measured and a health questionnaire was completed by the mother every two weeks. If the child was febrile (temperature $\geq 37.5^\circ C$), a blood film was made and examined immediately so that anti-malarial chemotherapy could be instituted if necessary. All children with fever and a parasite positive blood film were treated with a full course of chloroquine. As blood films from clinically well children were not read until some weeks later, asymptomatic infections were not treated. As free treatment was provided by study staff and was continuously available, we believe that very few infections were treated without our knowledge. Antimalarial chemoprophylaxis was not available to pregnant women in Prampram during the study period and thus transfer of anti-malarials across the placenta, or via breast milk, was estimated to be minimal. Infants did not receive malaria prophylaxis.

Parasite detection

Giemsa stained thick blood films were examined by oil immersion microscopy. The number of parasitised erythrocytes per 300 leucocytes was counted and the number of parasites per µl of blood calculated was based on an average leucocyte count of 13 000/µl in children under 1 year of age. Slides were classified as negative only after a minimum of 1000 leucocytes had been counted; the minimum parasite density that could reliably be detected by microscopy was 40 parasites/µl.

To improve the sensitivity of parasite detection, DNA was extracted from the red cell pellet and amplified using primers to the multicopy, subtelomeric 7H8/6 gene of P. falciparum, as described previously. This method had a resolution of approximately one parasite per µl, offering greater than 10-fold sensitivity over microscopy alone.

Examination of APOE genotype

DNA prepared from the red cell pellet contained also human DNA extracted from white blood cells trapped in the pellet, and so it was usable for determination of APOE genotypes. This was done using the method of Wenham et al.

Statistical analysis

The ages (in weeks) at which infection was detected were evaluated using the Kaplan-Meier survival analysis method in conjunction with the log rank test. This is a statistical procedure for estimating time to event models in the presence of differing follow up times and “censored” cases (that is, in this context, of infants who remained infection free at the termination of the study). Mean times to infection were compared between APOE genotypes.

The maximum parasite counts detected in each infected infant were compared using one way analyses of variance. As these counts were very positively skewed, a natural logarithmic transformation was applied to normalise the data distribution. Geometric mean maximum parasite densities were also compared between APOE genotypes and allele frequencies.

In the presentation of results, all summary statistics are accompanied by their 95% confidence limits.

RESULTS AND DISCUSSION

Infection occurred in 96 (87.3%) of the 110 infants during the study period. The mean time to infection was 48 weeks (95% confidence interval 41 to 55). Among the 110 children for whom an APOE genotype was obtained, the allele frequencies were 14.5%, 61.4%, and 28.5% for $e2$, $e3$, and $e4$, respectively. Overall, mean time to infection differed (statistically) significantly between genotypes (log rank test: $\chi^2(4)=15.69$, $p=0.008$), indicating that APOE genotype affects risk of malaria infection. A more detailed evaluation showed that the latter result was the result of the mean time to infection being significantly shorter for infants with a $e2/e2$ genotype compared to all other genotypes, but the differences between the remaining five genotypes were all non-significant (log rank test: $\chi^2(4)=2.94$, $p=0.568$). However, this result must be interpreted with great caution as only four infants had an $e2/e2$ genotype. Calculation of the expected frequency of APOE $e2$ homozygotes in this population, using the Hardy-Weinberg distribution, gives a value of only 2.6%, so clearly to evaluate our findings a much larger study would be needed to yield an adequate number of these homozygotes.

A secondary analysis was carried out based on the presence of a particular type of allele (table 1). No significant relationship was found between mean time to infection and the presence of a $e2$ allele (log rank test: $\chi^2(1)=0.48$, $p=0.491$), the presence of a $e3$ allele (log rank test: $\chi^2(1)=0.10$, $p=0.755$), or the presence of a $e4$ allele (log rank test: $\chi^2(1)<0.01$, $p=0.985$). Thus, if APOE $e2$ does confer a risk, possibly by affecting entry of sporozoites into the liver, it does so only in homozygotes.

To find if the number of maturing liver schizonts (and thus parasite density in the blood) depended on APOE genotype, we

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Mean time (in weeks) to infection by allele type/combo</th>
<th>Sample size</th>
<th>Number censored*</th>
<th>Mean time (weeks) to infection</th>
<th>95% confidence limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allele combination</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All infants</td>
<td>110</td>
<td>14</td>
<td>48</td>
<td>41 to 55</td>
<td></td>
</tr>
<tr>
<td>$e2/e2$</td>
<td>4</td>
<td>0</td>
<td>13</td>
<td>0 to 26</td>
<td></td>
</tr>
<tr>
<td>$e2/e3$</td>
<td>15</td>
<td>3</td>
<td>45</td>
<td>31 to 59</td>
<td></td>
</tr>
<tr>
<td>$e2/e4$</td>
<td>9</td>
<td>2</td>
<td>55</td>
<td>26 to 85</td>
<td></td>
</tr>
<tr>
<td>$e3/e3$</td>
<td>44</td>
<td>5</td>
<td>51</td>
<td>40 to 62</td>
<td></td>
</tr>
<tr>
<td>$e3/e4$</td>
<td>32</td>
<td>3</td>
<td>43</td>
<td>29 to 56</td>
<td></td>
</tr>
<tr>
<td>$e4/e4$</td>
<td>6</td>
<td>2</td>
<td>66</td>
<td>45 to 87</td>
<td></td>
</tr>
<tr>
<td>$e2$ absent</td>
<td>82</td>
<td>10</td>
<td>49</td>
<td>41 to 58</td>
<td></td>
</tr>
<tr>
<td>$e2$ present</td>
<td>28</td>
<td>4</td>
<td>44</td>
<td>31 to 57</td>
<td></td>
</tr>
<tr>
<td>$e3$ absent</td>
<td>19</td>
<td>3</td>
<td>51</td>
<td>32 to 69</td>
<td></td>
</tr>
<tr>
<td>$e3$ present</td>
<td>91</td>
<td>11</td>
<td>48</td>
<td>40 to 55</td>
<td></td>
</tr>
<tr>
<td>$e4$ absent</td>
<td>63</td>
<td>8</td>
<td>48</td>
<td>39 to 57</td>
<td></td>
</tr>
<tr>
<td>$e4$ present</td>
<td>47</td>
<td>6</td>
<td>48</td>
<td>37 to 59</td>
<td></td>
</tr>
</tbody>
</table>

*Number of children infection free at termination of study.
examined parasite density. In most of the infants, the density was very low, being detectable by PCR but not reliably so by microscopy. Values were mainly in the range of tens or hundreds of infected red blood cells per microlitre, with relatively few in the low thousands and only one very high value, 60 000/μl. Table 2 shows the (geometric) mean maximum parasite counts for each of the genotypes. The average parasite density was slightly lower for those infants who were APOE ε2/ε2 homozygotes, but overall the differences between the subgroups were not statistically significant (F(5,90) = 0.580, p=0.849) or the presence of an ε4 allele (F(1,94) = 0.883, p=0.350).

Table 2 Geometric mean maximum parasite count levels by allele type/combination

<table>
<thead>
<tr>
<th>Allele combination</th>
<th>Sample size</th>
<th>Geometric mean max parasite count</th>
<th>95% confidence limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>All infants</td>
<td>96</td>
<td>198</td>
<td>126 to 309</td>
</tr>
<tr>
<td>ε2/ε2</td>
<td>4</td>
<td>72</td>
<td>1 to 3647</td>
</tr>
<tr>
<td>ε2/ε3</td>
<td>12</td>
<td>303</td>
<td>117 to 783</td>
</tr>
<tr>
<td>ε2/ε4</td>
<td>8</td>
<td>229</td>
<td>43 to 1218</td>
</tr>
<tr>
<td>ε3/ε3</td>
<td>39</td>
<td>249</td>
<td>115 to 541</td>
</tr>
<tr>
<td>ε3/ε4</td>
<td>29</td>
<td>128</td>
<td>53 to 310</td>
</tr>
<tr>
<td>ε4/ε4</td>
<td>4</td>
<td>277</td>
<td>15 to 4994</td>
</tr>
<tr>
<td>ε2 absent</td>
<td>82</td>
<td>192</td>
<td>111 to 332</td>
</tr>
<tr>
<td>ε2 present</td>
<td>28</td>
<td>217</td>
<td>100 to 470</td>
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<tr>
<td>ε3 absent</td>
<td>19</td>
<td>179</td>
<td>61 to 525</td>
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<tr>
<td>ε3 present</td>
<td>91</td>
<td>202</td>
<td>122 to 333</td>
</tr>
<tr>
<td>ε4 absent</td>
<td>63</td>
<td>237</td>
<td>130 to 433</td>
</tr>
<tr>
<td>ε4 present</td>
<td>47</td>
<td>155</td>
<td>77 to 309</td>
</tr>
</tbody>
</table>

In summary, this study points to a possible correlation between susceptibility to malaria infection and homozygous carriage of the APOE ε2 allele in the study population. One interpretation of these data might be that early malaria infection is beneficial in the long term, as has been suggested by epidemiological studies, perhaps allowing children to acquire protective immune responses while being relatively protected from severe malaria by passively transferred maternal antibodies or by physiological factors. Clearly, further studies of the role of APOE in susceptibility to malaria are warranted using a larger numbers of infants, and in particular identifying more infants with an ε2/ε2 combination in order to improve the precision of the estimates of both time to infection and maximum parasite count.
counts. Also, it would be of interest to examine the APOE genotypes of older children or adults with much more severe disease, including cerebral malaria, to find out if APOE does in fact determine susceptibility to severity of damage caused by the protozoan.

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