Effect of repeated infestations of BALB/c mice with Ixodes ricinus nymphs on tick-borne encephalitis virus infection

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Abstract. The effect of repeated infestations of BALB/c mice with Ixodes ricinus (L.) nymphs on tick borne encephalitis (TBE) virus infection was studied. Enhancement of nymphal feeding, occurring in noninfected mice during the quaternary infestations, was less apparent or absent in female nymphs engorged on TBE virus infected mice. The mice infected with TBE virus during quaternary tick infestation survived significantly longer (P < 0.01) than mice infected with TBE virus during the primary tick infestation. The mean titre of virus in murine blood (determined by plaque assay) was significantly lower (P < 0.01) and the number of nymphs acquiring virus was reduced (P < 0.05) when feeding on hosts infected during the quaternary infestation. The results indicate that repeated infestations of I. ricinus nymphs on BALB/c mice, although enhancing tick feeding, reduced infection with TBE virus when inoculated intraperitoneally.

It has been suggested that some vectors and pathogens share similar antigen determinants, and several authors have demonstrated that host anti-tick immunoresponse crossreacted with protozoans, bacteria, and virus (Francis and Little 1964, Callow and Steward 1978, Bell et al. 1979, Wikel 1980, Heller-Haupt et al. 1983, Eckblad et al. 1984, Brossard and Moreau 1985, Fivaz et al. 1989, Rubaire-Akiiki 1990). Grubhofer et al. (1990) observed the interaction of tick-borne encephalitis (TBE) virus protein E with some labelled lectins and the same author (Grubhofer et al. 1991) described sugar-binding lectins from haemolymph of Ixodes ricinus (L.) and from three other species of soft ticks. He postulated that these tick lectins play an important role in the process of self-non-self recognition and the defence reaction in ticks, influencing also the multiplication of viruses in ticks.

Belorussian authors (Votyakov 1980, Votyakov and Mishaeva 1980, 1990, Mishaeva 1990) published several papers on the possible interactions between acquired immunity of hosts to ticks and TBE virus. In their experiments, the titres of TBE virus in blood were significantly lowered and the susceptibility to infection was reduced in mice, rabbits and guinea pigs passively or actively (by natural tick feeding or by antigen injections) immunized against Dermacentor andersoni Stiles and Ixodes ricinus (L.) when compared with control naive animals. The number of ticks that acquired virus during co-feeding with infected ticks was also decreased in hosts immunized against ticks. A similar interaction was observed by Jones and Nuttall (1990). In their experiments with Thogoto virus and Rhipicephalus appendiculatus Neumann, the number of recipient ticks that acquired virus during co-feeding with infected donor ticks was significantly reduced on resistant guinea-pigs when compared with naive hosts.

Recently Labuda et al. (1993a) described an enhancement of TBE virus transmission in R. appendiculatus nymphs by salivary gland extracts of partially fed females of R. appendiculatus, Dermacentor reticulatus (Fabricius) and I. ricinus. These results indicated that a comparable mechanism of saliva activated transmission (SAT) (Jones et al. 1989, Nuttall and Jones 1991) operated in the transmission of TBE virus, predominantly modulating the skin site at the tick attachment (Nuttall et al. 1994).

Dushábek et al. (1995) ascertained that repeated infestations of BALB/c mice with Ixodes ricinus nymphs induced significant suppression of the murine immune system and enhancement of nymphal feeding during four successive infestations. The suppression of B-cell competence and antibody generation, together with the decrease in skin mast cell numbers during tertiary infestation in the skin at the tick attachment sites, were considered to be responsible for impairing the host defence mechanisms directed against tick feeding.

The present paper examined the effect of immunosuppression in BALB/c mice caused by repeated infestations with I. ricinus nymphs on TBE virus multiplication and evaluated the infectivity and feeding ability of larval and nymphal ticks on infected and non-infected suppressed and naive hosts.
MATERIALS AND METHODS

Nymphs and larvae of *Ixodes ricinus* (L.) ticks originating from a pathogen free laboratory colony were used for the experiments. Thirty female BALB/c mice (ANLAB Ltd., Charles River Wiga GmbH, Germany) were fourfold infected at two-week intervals each with 10 nymphs (30–60 days after molting), receiving the first infestation at the age of 10–12 weeks. Approximately fifty 30-day-old larvae were added to each mice during the quaternary nymphal infestation. Additional 30 age-matched naive female mice were infected each with 10 nymphs and eca 50 larvae and used as the primary control parallel to the quaternary infestations.

To eliminate the effect of the SAT factor at the tick attachment site, and to study predominantly the effect of suppression of anti-tick-feeding antibody response on TBE virus infections in BALB/c mice, TBE virus was injected intraperitoneally. Highly intracerebrally passaged (approx. 50 passages) Czech prototype TBE virus strain Hypr was used for the infection of mice. The invasivity index defined as the difference between log$_{10}$LD$_{50}$ after intracerebral and subcutaneous inoculation was 1.8. Thirteen mice of the quaternary infested experimental group, fifteen mice of the primarily infested control group and ten tick-free mice were injected intraperitoneally with 0.25 ml of 1 × 10$^4$ PFU (plaque forming units) suspension of the virus in L-15 medium supplemented with 3% fetal bovine serum (FBS) at the first day of tick infestations. The survival of infected mice in days, the titres of viraemia 3 days p.i., and feeding parameters of larval and nymphal ticks on experimental and control mice were recorded. Larvae and nymphs engorged on infected mice were examined for virus presence two weeks after detachment. Individual nymphs and larvae were homogenized by mortar and pestle in 0.2 ml L-15 medium supplemented with 3% fetal bovine serum and 200 units penicillin, 200 µg streptomycin and 5 µg amphotericin B per 1 ml. TBE virus was assayed by the plaque method in PS cells using the technique by De Madrid and Porterfield (1969).

Engorged nymphs were weighed and reared to molting into adults for sex determination. The sex of nymphs used for virus detection was determined according to the engorged weight (Belozerov et al. 1993). Nymphs weighing less than 3.5 mg were considered to be male nymphs, and those exceeding 3.5 mg were considered female nymphs.

RESULTS

Feeding parameters for *Ixodes ricinus* nymphs engorged on TBE virus infected and non-infected BALB/c mice are shown in Table 1. No significant differences (P > 0.05) were found in tick yield, in the length of the feeding period as well as in the engorged weight in male nymphs engorged, in either infected or non-infected mice, within primary or quaternary infestation. However, these parameters significantly differed when the results of the primary infestation were compared with those of the quaternary infestation. Tick yield (P < 0.05) and male nymph engorged weight (P < 0.01) were higher and feeding period (P < 0.01) was shorter in ticks engorged on quaternarily infested mice.

In female nymphs, differences were recorded between those engorged on infected and non-infected mice. The feeding period was longer in female nymphs engorged on non-infected mice during the primary infestation, but shorter during the quaternary infestation (P < 0.01). Engorged weight of female nymphs was lower when feeding on non-infected mice during the primary infestation (P < 0.05) and higher during the quaternary infestation (P < 0.01). Therefore, the enhanced feeding success during the quaternary infestation was less apparent in female nymphs engorged on mice infected with TBE virus. The same trend also occurred in the quaternary feeding parameters of male nymphs, but these differences were not significant (P > 0.05).

No differences were found in larval feeding between infected and non-infected mice during primary and quaternary infestations (P > 0.05) (Table 2). However, the yield of larvae was higher (P < 0.01), the engorged weight was lower (P < 0.01), and the feeding period was shorter (P < 0.05) in larvae feeding on quaternarily infested hosts.

The mean titres of virus (PFU/ml) 3 days p.i. in tick-free mice and those receiving the primary tick infestations were higher than virus titres in quaternarily infested mice. However, these differences were not significant (P > 0.05) (Table 3). The quaternarily infested mice infected with TBE virus survived significantly longer (P < 0.01) than mice primarily infected by ticks. Although the percentage of ticks infected by feeding (seven male and two female nymphs) was low (5.19% and 1.62%), the number of infected ticks from primary infestation was significantly higher (P < 0.05) than from the quaternary infestation. The mean dose of virus (PFU/ml) detected in nymphs two weeks after detachment from infected hosts was significantly higher (P < 0.01) in nymphs engorged on primarily infested mice than in nymphs from quaternarily infested hosts. Of these, seven were male nymphs and two were female nymphs. Only one larva was infected by TBE virus when engorged on infected, primarily infested host, as detected in plaque assay.

DISCUSSION

Repeated infestations of BALB/c mice with nymphs of *Ixodes ricinus* in our previous experiments (Dusdbé et al. 1995) resulted in the enhanced feeding success of nymphal ticks, manifested by increased tick yield, increased tick engorged weight, and shortened feeding period. Tick saliva negatively influenced the host's immune system, inducing both suppression of

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Table 1. Feeding parameters of *Ixodes ricinus* nymphs engorged on BALB/c mice infected with TBE virus during the primary and quaternary infestations. Means ± standard errors (SE). Means followed by the same letters are not significantly different (P > 0.05).

<table>
<thead>
<tr>
<th>Infestation</th>
<th>Primary</th>
<th></th>
<th>Quaternary</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Infected</td>
<td>Non-infected</td>
<td>Infected</td>
<td>Non-infected</td>
</tr>
<tr>
<td>Number of mice</td>
<td>15</td>
<td>15</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>Number of nymphs</td>
<td>150</td>
<td>150</td>
<td>130</td>
<td>130</td>
</tr>
<tr>
<td>Tick yield (Σ)</td>
<td>135</td>
<td>138</td>
<td>124</td>
<td>127</td>
</tr>
<tr>
<td>Tick yield/mouse</td>
<td>9.00±1.74</td>
<td>9.20±1.42</td>
<td>9.54±0.91</td>
<td>9.77±0.91</td>
</tr>
<tr>
<td>Feeding period (days)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>male nymphs</td>
<td>4.46±1.41</td>
<td>4.08±0.76</td>
<td>3.66±1.04</td>
<td>3.56±0.82</td>
</tr>
<tr>
<td>female nymphs</td>
<td>4.20±0.81</td>
<td>4.65±0.93</td>
<td>4.24±1.13</td>
<td>3.81±0.74</td>
</tr>
<tr>
<td>Engorged weight (mg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>male nymphs</td>
<td>2.64±0.61</td>
<td>2.65±0.38</td>
<td>2.81±0.32</td>
<td>2.88±0.29</td>
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<tr>
<td>female nymphs</td>
<td>4.76±0.48</td>
<td>4.60±0.51</td>
<td>4.57±0.52</td>
<td>4.82±0.42</td>
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</table>

Table 2. Feeding parameters of *Ixodes ricinus* larvae engorged on BALB/c mice infected with TBE virus during primary and quaternary nympha infestations. Means ± standard errors (SE). Means followed by the same letters are not significantly different (P > 0.05).

<table>
<thead>
<tr>
<th>Infestation</th>
<th>Primary</th>
<th></th>
<th>Quaternary</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Infected</td>
<td>Non-infected</td>
<td>Infected</td>
<td>Non-infected</td>
</tr>
<tr>
<td>Number of mice</td>
<td>15</td>
<td>15</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>Tick yield (Σ)</td>
<td>163</td>
<td>125</td>
<td>190</td>
<td>141</td>
</tr>
<tr>
<td>Tick alive (Σ)</td>
<td>88</td>
<td>58</td>
<td>147</td>
<td>102</td>
</tr>
<tr>
<td>Tick alive (%)</td>
<td>54.0±</td>
<td>46.4±</td>
<td>74.4±</td>
<td>72.3±</td>
</tr>
<tr>
<td>Feeding period (days)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.17±0.46</td>
<td>3.31±0.66</td>
<td>3.08±0.30</td>
<td>3.10±0.39</td>
</tr>
<tr>
<td>Engorged weight (mg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.46±0.12</td>
<td>0.47±0.05</td>
<td>0.42±0.04</td>
<td>0.41±0.03</td>
</tr>
</tbody>
</table>

Table 3. Infectivity of TBE virus for BALB/c mice and ability to acquire virus by feeding larvae and nymphs of *Ixodes ricinus* during primary and quaternary nympha infestations. Dose of virus: 0.25 ml of 1 × 10⁶ PFU suspension/mice. Data followed by the same letters are not significantly different (P > 0.05).

<table>
<thead>
<tr>
<th>Infestation</th>
<th>None</th>
<th>Primary</th>
<th>Secondary</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of mice infected</td>
<td>10</td>
<td>15</td>
<td>13</td>
</tr>
<tr>
<td>Mean viraemia, day 3 p.i. (PFU/ml)</td>
<td>1.60 × 10³</td>
<td>2.20 × 10³</td>
<td>6.20 × 10²</td>
</tr>
<tr>
<td>Time of survival (days)</td>
<td>7.70±0.51</td>
<td>7.33±0.51</td>
<td>7.85±0.39</td>
</tr>
<tr>
<td>Number of nymphs engaged</td>
<td>135</td>
<td>124</td>
<td></td>
</tr>
<tr>
<td>Number of nymphs infected</td>
<td>7±</td>
<td>2±</td>
<td></td>
</tr>
<tr>
<td>% of nymphs infected</td>
<td>5.19±</td>
<td>1.62±</td>
<td></td>
</tr>
<tr>
<td>Mean dose of virus in nymphs (PFU/ml)</td>
<td>2.60 × 10⁴</td>
<td>5.30 × 10⁴</td>
<td></td>
</tr>
<tr>
<td>Number of larvae engaged</td>
<td>88</td>
<td>147</td>
<td></td>
</tr>
<tr>
<td>Number of larvae infected</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Dose of virus in larva (PFU/ml)</td>
<td>5.40 × 10³</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

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B-cell competence and specific anti-tick antibody generation, as well as reducing the numbers of skin mast cells in tick attachment sites during the tertiary infestation. The feeding of male nymphs was also enhanced in the present experiments and it was not significantly influenced by murine infection with TBE virus. However, there were differences in female nymphal feeding between TBE virus-infected and non-infected mice in their engorged weight and length of the feeding period, which indicates a less feeding success in female nymphs during the quaternary infestations on virus infected mice. As the blood intake of female nymphs is considerably greater than male nymphs or larvae, the influence of murine TBE virus infection on female nymph feeding parameters may be manifested more intensively than in male nymphs or larvae.

It is generally considered that the vertical transmission of TBE virus does not result in significant amplification of TBE virus infection at the tick population level (Reháček 1965, Burgdorfer and Varma 1967) and the trans-ovarial transmission of TBE virus seems to be rather scarce in *Ixodes ricinus* ticks (Danielová and Holubová 1991, Labuda et al. 1993b). Although trans-stadial transmission of TBE virus occurs more frequently in *R. appendiculatus*, an unnatural vector of TBE virus, the detection of TBE virus in newly moulted *I. ricinus* ticks is difficult to assess by PFU assay, mostly due to an eclipse phase after metamorphosis during which the virus cannot be detected in the vector (Riedl et al. 1973, Liebisch 1978, Labuda et al. 1993b). This is probably the main reason for the low percentages of virus detection in nymphs two weeks after feeding on viraemic hosts in our experiments. Despite this fact, the number of nymphs infected by feeding on quaternarily infested viraemic hosts and the mean dose of virus identified in these nymphs (PFU/ml) were significantly lower than in nymphs feeding on primarily infested hosts. These phenomena are accompanied by a significantly longer survival of virally-infected mice which had been repeatedly infested with nymphal ticks.

There was not any apparent relationship between weight and virus titres in nymphal ticks, because only two heavy female nymphs but eight light male nymphs acquired the virus during feeding on viraemic hosts. Similar results were reported by Linthicum and Logan (1994) during feeding of *Hyalomma truncatum* Koch on guinea pigs infected by Venezuelan equine encephalitis virus. However, Davies et al. (1986) reported that the amount of Thogoto virus ingested was proportional to weight gain of *R. appendiculatus* engorged on viraemic hamsters.

Our results showed that repeated infestations of BALB/c mice with *Ixodes ricinus* nymphs influenced negatively the TBE virus multiplication in hosts, despite the enhancement of tick feeding and its negative influence on murine immune system. This was manifested by prolonged survival of repeatedly infested mice, fewer ticks infected with virus following feeding on infected mice and smaller doses of virus acquired by ticks during this feeding. This is in accordance with results of Jones and Nuttall (1990) and of Belorusssian authors (Votyakov 1980, Votyakov and Mishaeva 1980, 1990, Mishaeva 1990) who demonstrated the effect of repeated tick infestation on transmission of Thogoto and TBE viruses. As this phenomenon was apparent also after intraperitoneal TBE virus inoculations, which eliminated the direct influence of SAT factor on the virus at the tick attachment sites, the induction of systemic anti-viral immune responses by tick feeding should also be considered.

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REFERENCES


