Tick salivary gland extracts promote virus growth *in vitro*

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SUMMARY

Saliva of blood-feeding arthropods promotes infection by the vector-borne pathogens they transmit. To investigate this phenomenon *in vitro*, cultures of mouse L cells were treated with a salivary gland extract (SGE) prepared from feeding ticks and then infected with vesicular stomatitis virus (VSV). At low input doses of VSV, viral yield was increased 100-fold to 10000-fold by 16–23 h post-infection compared with untreated cultures, and depending on the SGE concentration. SGE-mediated acceleration of viral yield corresponded with the earlier appearance of VSV nucleocapsid protein as detected by 2-dimensional electrophoresis of infected cells. The observation that physiological doses of virus (i.e. doses likely to be inoculated by an infected arthropod vector into its vertebrate host during blood-feeding) respond to SGE treatment *in vitro* provides a new opportunity for identifying the factors in tick saliva that promote virus transmission *in vivo*.

Key words: saliva-activated transmission, tick salivary gland, *Dermacentor reticulatus*, *Rhipicephalus appendiculatus*, vesicular stomatitis virus.

INTRODUCTION

Increasing evidence indicates that the transmission of pathogens by ticks is independent of the development of a systemic infection. For example, tick-borne encephalitis virus was transmitted from infected to uninfected ticks feeding together on non-viraemic natural hosts, and even occurred when the vertebrate hosts were immune to the virus (Labuda *et al*. 1993*b*, 1997); non-systemic transmission of *Borrelia burgdorferi*, the bacterial agent of Lyme disease, has been reported for laboratory and natural (sheep) hosts (Gern & Rais, 1996; Ogden, Nuttall & Randolph, 1997). The phenomenon underlying non-systemic transmission is believed to be saliva-activated transmission (SAT), the exploitation of arthropod saliva components that modulate the host response to arthropod bites (Nuttall & Jones, 1991). These components have anti-haemostatic, anti-inflammatory, and immunosuppressive properties (Wikel, 1996). The combined effect of such bioactive saliva components on the host facilitates blood-feeding. Pathogens transmitted by blood-sucking arthropods appear to exploit the modulatory effects of their vectors' saliva thereby promoting their transmission. Most examples of SAT have been demonstrated with arthropod-borne viruses (arboviruses) transmitted by ticks, such as Thogoto virus (Jones, Hodgson & Nuttall, 1989) and tick-borne encephalitis virus (Labuda *et al*. 1993*a*). However, the observations that *Leishmania* infectivity in animal models was enhanced by salivary gland products from sandflies (Titus & Ribeiro, 1988; Samuelson *et al*. 1991), and that bovine lymphocyte susceptibility to *Theileria parva* sporozoite infection *in vitro* was increased by preincubation with tick salivary gland extracts (Shaw, Tilney & McKeever, 1993), indicate that SAT is a more general phenomenon.

To determine whether SAT underlies non-systemic transmission requires identification of the SAT factor(s). Hitherto, SAT has been demonstrated *in vivo* but not *in vitro*. Here we describe an *in vitro* model using vesicular stomatitis virus (VSV), a well-characterized virus that is commonly used to investigate factors affecting viral growth (Baer, Bellini & Fishbein, 1990). Because of the difficulty in obtaining sufficient tick saliva, salivary gland extract (SGE) was used as it shows a similar SAT activity profile to tick saliva (Jones, Kaufman & Nuttall, 1992).

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MATERIALS AND METHODS

Cell cultures and virus

The continuous fibroblast line of mouse L929 cells (L cells), originating from the Imperial Cancer

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Research Fund Laboratory (London, UK), was routinely passaged in Leibowitz L-15 medium supplemented with 5% bovine calf serum at 37 °C, and used to propagate VSV (Indiana strain). Virus titre was expressed as the tissue culture infectious dose that resulted in a cytopathic effect 48 h p.i. in 50% cells ($TCID_{50}$) using 4 replicates per virus dilution. Cultures were fixed, then stained with crystal violet and the results read at 542 nm using an ELISA plate reader.

Preparation of tick salivary gland extract (SGE)

Dermacentor reticulatus ticks were collected by flagging the vegetation in selected localities of western Slovakia known to be free of tick-borne encephalitis virus. *Rhipicephalus appendiculatus* ticks were obtained from a laboratory colony (Jones *et al*. 1988). Adult ticks were allowed to feed on Balb/C female mice, as described previously (Labuda *et al*. 1996). At day 5 of feeding (the period of maximal SAT activity), female ticks were gently removed from the mice and their salivary glands dissected out on ice, washed with phosphate-buffered saline (PBS), and homogenized in pools of salivary glands from 15 ticks in a final volume of 500 μl of PBS. The protein concentration of the clarified supernatant (1·12 mg total protein/ml) was determined using the Bio-Rad Protein Assay Kit samples (designated SGED5) were dried using a Speed-Vac, stored at 4 °C, and rehydrated prior to use.

Viral replication in the presence of SGE

Confluent monolayers of L cells grown in 1 ml of medium in a 24-well plate (approximately 4×10^5 cells/well) were treated with 25 μl of *D. reticulatus* SGED5 (final dilution 1:40), or either 25 μl or 50 μl of *R. appendiculatus* SGED5 (final dilution 1:40 or 1:20, respectively). After 24 h incubation at 37 °C, the medium was replaced with 1 ml of fresh medium containing 5 $TCID_{50}$ virus. Following 1 h of adsorption, the medium was replaced with fresh medium. At set time-intervals, 0·1 ml aliquots were harvested and replaced with fresh medium. The aliquots were assayed for infectivity by titration in monolayers of L cells as described above.

Viral protein synthesis in the presence of SGE

Following infection of L cells with VSV as described above, the medium was replaced with 2 ml of fresh medium containing 1 MBq of ^{14}C-labelled protein hydrolysate (UVVVR, Prague). After a further 19 h incubation, cell monolayers were prepared for isoelectric focusing (IEF) by washing 3 times with PBS and solubilization in 100 μl of buffer containing 9 M urea and 4% Triton X-100, followed by incubation at room temperature for 2 h, as described (Dunbar, 1987). Samples were then centrifuged at 50000 ***g*** for 2 h and the supernatants stored at −70 °C until use. Isoelectric focusing and polyacrylamide gel electrophoresis were performed using an LKB 2001 unit. Briefly, 105 mm (length) × 1·5 mm (diameter) gels were prepared with 2% ampholines pH 3–10 (Servalyte). Then 25 μl of each sample containing 2% β-mercaptoethanol and 2% ampholines were loaded per gel lane. Samples were focused at 12 kV/h. After IEF, gels were equilibrated in buffer containing 0·0625 M Tris–HCl, pH 6·8, 2% SDS, 5% β-mercaptoethanol and 10% glycerol. The second dimension was performed in sodium dodecyl sulphate (SDS) polyacrylamide gels with a linear gradient of 7·5–12·5% acrylamide. Following electrophoretic separation, using bromphenol blue as a marker, the gels were impregnated with PPO scintillator and dried. The dried gels were exposed to Du Pont X-ray films at −70 °C for 6 weeks and then photographed (Fig. 3) and examined using a Bio-Rad Image Analysis System (Fig. 4).

RESULTS

At 16 and 23 h post-infection (p.i.) the yield of VSV from cells treated with SGE from *D. reticulatus* ticks was approximately 100 times greater than the corresponding yields from control cells treated with PBS (Fig. 1). During the period 23 to 48 h p.i., the rate of viral replication in control cells exceeded that in SGE-treated cells. This was probably because, by 23 h p.i., most of the cells in the SGE-treated cultures had been lysed as a result of viral replication and consequently the cultures were unable to support further rounds of viral replication. Similar results were observed when L cells were treated with SGE prepared from *R. appendiculatus* ticks. At 20 h p.i., a SGE dose-dependent increase in viral yield was observed with viral titres raised by up to 10000-fold (Fig. 2). The effect of tick SGE on VSV replication was only observed at 5 $TCID_{50}$/ml. When the virus inoculum was > 10 $TCID_{50}$/ml, the rate of viral replication was unchanged compared with the controls (data not shown).

To determine whether the effect of tick SGE on VSV replication kinetics was detectable at the level of viral protein synthesis, radio-isotope labelled infected cells with or without treatment with *D. reticulatus*-derived SGE, were examined using 2-dimensional gel electrophoresis. First, to identify viral proteins, untreated cells were infected with 50 $TCID_{50}$/ml and examined 26 h p.i. The viral nucleocapsid (N) protein, phosphoprotein (P) and glycoprotein (G), were readily detectable based on their respective relative molecular weights and pI (Fig. 3A; compare uninfected cells shown in Fig. 3B). Secondly, untreated cells were infected with 5

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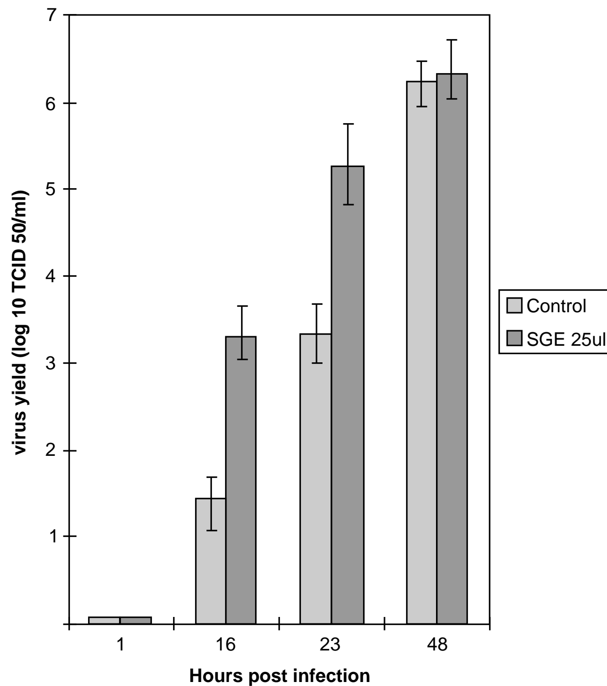

Fig. 1. Comparison of the growth kinetics of VSV in mouse L cell cultures pre-treated with either SGED5 of *Dermacentor reticulatus* ticks (25 μl) or PBS (control cells). Vertical line indicates standard error.

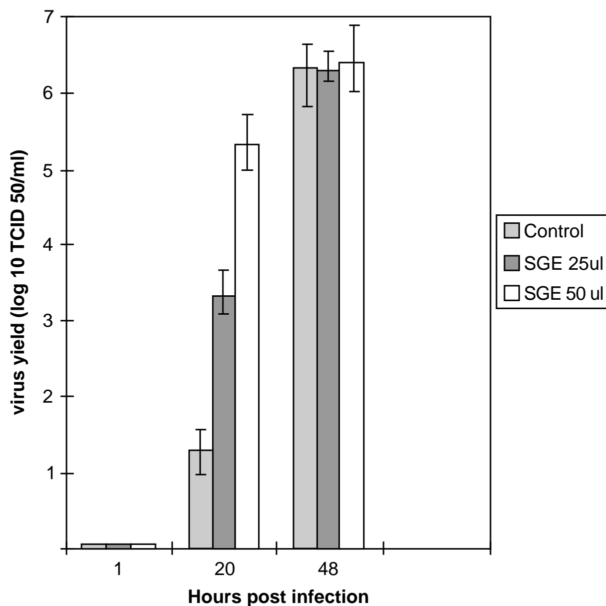

Fig. 2. Comparison of the growth kinetics of VSV in mouse L cell cultures pre-treated with either SGED5 of *Rhipicephalus appendiculatus* ticks (25 or 50 μl) or PBS (control cells). Vertical line indicates standard error.

$TCID_{50}$/ml; the N protein was not discernible at 19 h p.i. (Fig. 3C) but was apparent at 26 h (Fig. 3D). Based on these observations, the N protein, which plays a critical role in VSV replication (Banerjee & Barik, 1992), was selected as a marker to determine whether SGE treatment had an accelerative effect on viral protein synthesis.

The effect of SGE treatment on VSV N protein synthesis was examined by incubating confluent monolayers of L cells with *D. reticulatus*-derived SGE for 24 h prior to infection with 5 $TCID_{50}$/ml. At 19 h p.i., the N protein was clearly discernible (Fig. 4A). In contrast, at 19 h p.i., the pattern obtained with untreated infected cells (Fig. 4B) was similar to that of untreated uninfected cells (Fig. 4C), and VSV N protein was not apparent. There were no discernible differences between SGE-treated and untreated L cells (data not shown). SGE treatment of cells prior to VSV infection accelerated the production of N protein synthesis during the period when SGE-mediated acceleration of virus growth was detected (Figs 1 and 2). Thus the earlier detection of N protein was consistent with the increased virus infectivity titre, when cells were pre-treated with SGE.

DISCUSSION

Speculation about the mechanism of SAT of arboviruses has prompted experiments to analyse the immunomodulative effects of tick saliva. *In vitro* studies demonstrated that SGE derived from partially fed *D. reticulatus* ticks suppressed natural killer (NK) cell activity and depressed the stimulatory effect of interferon α on NK cell activity (Kubes *et al*. 1994). NK cells are known to have anti-viral activity hence the suppressive effect of tick SGE may contribute to SAT. Furthermore, ticks (including *D. reticulatus*) manipulate their hosts' cytokine network, which may account for their effect on NK activity (Fuchsberger *et al*. 1995); one of the group of cytokines affected are the interferons which also have anti-viral activity (Wikel, 1996).

Although the above findings support the hypothesis that the immunomodulative effect of tick saliva is responsible for SAT, direct evidence of an effect on viral infections *in vitro* has not been demonstrated hitherto. As VSV infection of L cells is a commonly used *in vitro* model, we tested the effect of SGE in this system. The results reported here provide evidence that tick saliva is able to promote virus replication *in vitro* as measured by 2 different methods. Furthermore, the *in vitro* effect of tick SGE on an arbovirus (VSV) that is not known to be transmitted by ticks supports the hypothesis that the immunomodulating activity underlying SAT is designed to promote tick blood-feeding, rather than being a special tick saliva–arbovirus interaction. The mechanism by which *D. reticulatus* SGED5 promoted VSV replication *in vitro* is undetermined. Possibly, low-level constitutive expression by the mouse cells of an anti-viral factor (e.g. interferon) was suppressed, or the anti-viral action was blocked, by treatment of the cells with tick SGE. The concomitant increase in the rate of N protein

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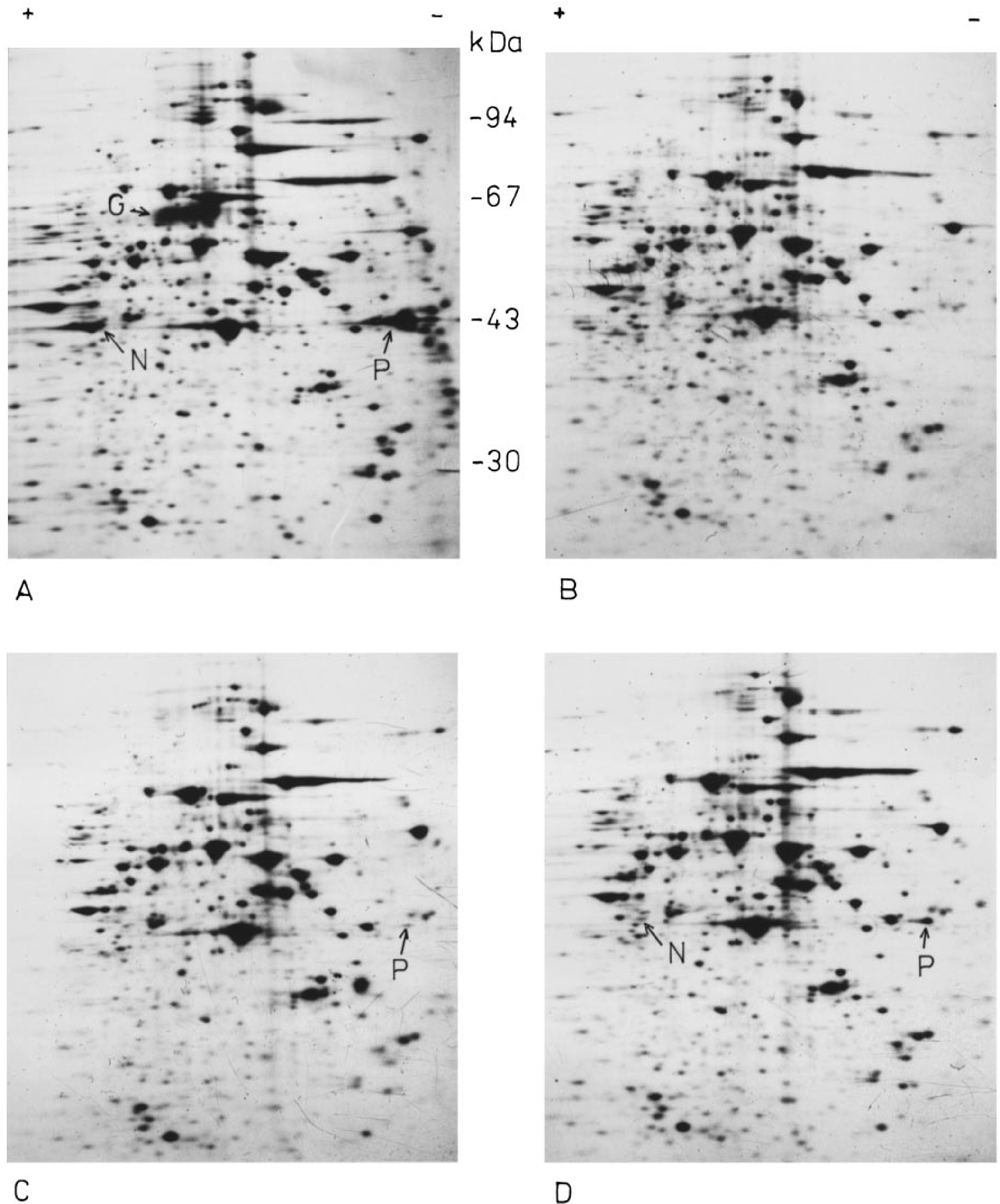

Fig. 3. Two-dimensional gel electrophoresis protein patterns of VSV-infected mouse L cells not treated with tick SGE. (A) Infected with 50 $TCID_{50}$ VSV or (B) uninfected and examined 26 h p.i. (C) and (D) Infected with 5 $TCID_{50}$ VSV and examined 19 and 26 h p.i. respectively. Photographs of developed gels.

accumulation may explain the increased viral yield as, in VSV replication, encapsidation is believed to require large stoichiometric quantities of N protein (Banerjee & Barik, 1992).

Extrapolation of the *in vitro* observations to the *in vivo* situation is not yet possible. However, these new *in vitro* findings, together with data from studies *in vivo* (Jones *et al.* 1992; Labuda *et al.* 1996),

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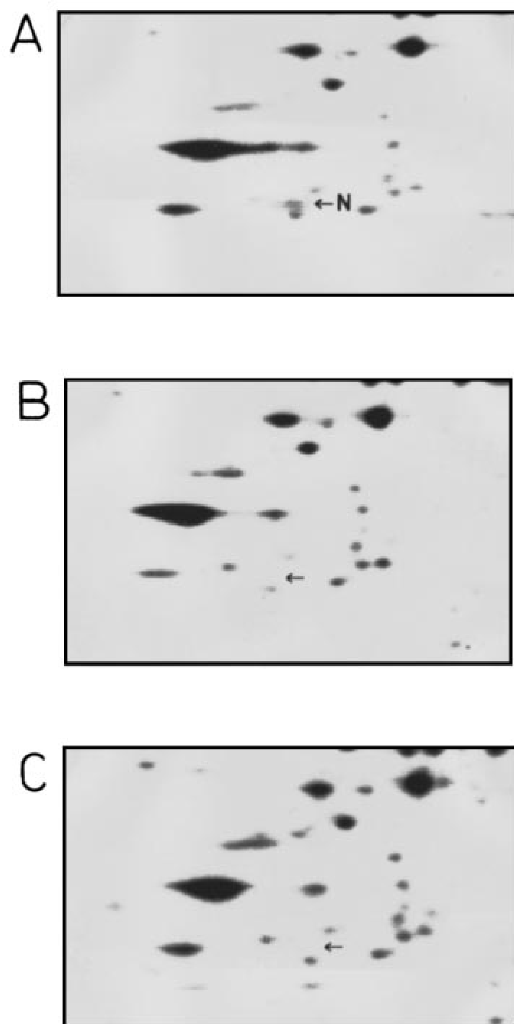

Fig. 4. Effect of tick SGE treatment on VSV N protein accumulation in mouse L cells at 19 h p.i. (A) Pre-treated with SGED5 and then infected with 5 $TCID_{50}$ VSV; (B) untreated cells infected with 5 $TCID_{50}$ VSV; (C) uninfected cells pre-treated with SGED5. Arrow indicates the position of VSV N protein. Images were generated using the BioRad Molecular Analyst Software Cat. No. 170-7560.

suggest that localized changes induced by tick saliva, which may allow $\geqslant$ 10-fold increase in virus multiplication within the tick 'bite' site, could be critical to the transmission (and hence survival) of the virus in Nature. Such an effect of tick saliva on virus transmission is consistent with the higher relative reproductive number, R_o, for non-systemic compared with systemic transmission of tick-borne encephalitis virus (Randolph, Gern & Nuttall, 1996). In this context, it is also significant that the effect of SGE *in vitro* was subtle, requiring a carefully equilibrated system (i.e. very low virus dose) to observe the effect. This observation is not surprising, considering the natural infection processes *in vivo*: (i) ticks secrete repeated 'pulses' of only a few infectious virus particles during feeding (Kaufman & Nuttall, 1996) and (ii) the immunomodulative effect of tick saliva need only be directed against a relatively small area of the host, sufficient to ensure the successful feeding of the tick and survival of the host. Moreover, unlike the *in vitro* model in which cells were treated only once with SGE, *in vivo* the effect of tick saliva on cells within the feeding site is probably continuous during virus transmission and localized cell infection. The development of an *in vitro* system provides a relatively simple assay (compared with tick infestation of animals) for identifying the mechanism and active saliva component(s) that promote non-systemic transmission of arboviruses by ticks.

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