

# Mosquito feeding modulates Th1 and Th2 cytokines in flavivirus susceptible mice: an effect mimicked by injection of sialokinins, but not demonstrated in flavivirus resistant mice

NORDIN S. ZEIDNER<sup>1</sup>, STEPHEN HIGGS<sup>2</sup>, CHRISTINE M. HAPP<sup>1</sup>, BARRY J. BEATY<sup>2</sup> & BARRY R. MILLER<sup>1</sup>

<sup>1</sup>Division of Vector-Borne Infectious Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, PO Box 2087, Foothills Campus, Fort Collins, CO 80522, USA

<sup>2</sup>Arthropod-Borne and Infectious Diseases Laboratory, Department of Microbiology, Colorado State University, Fort Collins, CO 80523-1682, USA

## SUMMARY

*Culex pipiens* and *Aedes aegypti* mosquitoes were fed on C3H/HeJ mice and systemic cytokine production was quantified from stimulated lymphocytes harvested four to ten days after feeding. Mosquito feeding on C3H/HeJ mice significantly down regulated IFN $\gamma$  production seven to ten days post feeding by *Cx. pipiens* and seven days after *Ae. aegypti* feeding. Th2 cytokines, IL-4 and IL-10, were significantly up regulated 4–7 days after *Cx. pipiens* and *Ae. aegypti* feeding. The immunosuppressive effect of *Cx. pipiens* feeding on systemic cytokine production was not evident in congenic flavivirus resistant (C3H/RV) mice, as systemic IFN $\gamma$  and IL-2 were significantly up regulated at days 7 and 10, correlating with a significant decrease in IL-4 10 days after feeding by *Cx. pipiens* mosquitoes. Inoculation of 5–1000 ng of sialokinin-I into C3H/HeJ mice mimicked the effect of *Ae. aegypti* feeding by down regulating Th1 cytokines and significantly up regulating Th2 cytokines four days post inoculation. Injections of sialokinin-II resulted in only moderate effects on IFN $\gamma$  and IL-4 production seven and ten days after injection. Thus natural feeding by two arbovirus vectors had a profound T cell modulatory effect in vivo in virus susceptible animals which was not demonstrated in the flavivirus resistant host. Moreover, sialokinin-I and sialokinin-II mimicked the effect of mosquito feeding by modulating the host T cell response. These results may lend new insight into specific aspects of the role of the mosquito vector in potentiating virus transmission in the mammalian host.

**Keywords** cytokines, IFN $\gamma$ , IL-4, flavivirus, flavivirus resistant mice

## INTRODUCTION

Flaviviridae, long recognized as the most significant family of arboviruses in terms of their impact on human health, encompass three of the most important arthropod-borne viral infections of humankind, dengue fever, yellow fever and Japanese encephalitis (Monath & Heinz 1996). These arboviral infections currently induce significant morbidity and mortality throughout the world with the probability of increased incidence concurrent with changes in global climate (Reeves *et al.* 1994, Gubler 1996, Jetten & Focks 1997). Arboviruses are vectored by mosquitoes, the most important of which are the *Culex* and *Aedes* species (Karabatsos 1985). The role of vectors, and in particular the significance of vector feeding in actively promoting transmission of pathogenic arboviruses, has been recognized but is still poorly characterized (Jones *et al.* 1990, Osorio *et al.* 1996).

Vector saliva contains numerous pharmacologic agents, producing a myriad of systemic effects in the mammalian host (Wikel 1982, Ribeiro 1987, Champagne 1994). Salivary gland apyrases, shown to be secreted in saliva by anopheline as well as culicine mosquitoes (Ribeiro 1984b, 1985), inhibit ADP-induced platelet aggregation, limit local blood coagulation and promote successful feeding by decreasing the amount of time mosquitoes spend probing for microvasculature on the host (Ribeiro 1984a). Likewise, two novel tachykinins, sialokinin-I and sialokinin-II, have been isolated from the salivary gland of *Ae. aegypti* (Champagne 1994). These neuropeptides have been recently sequenced and shown to have smooth muscle contracting activity similar to mammalian tachykinin, while sharing amino acid homology with mammalian substance P, a potent vasodilator (Champagne 1994). Mosquito tachykinins are highly conserved among anopheline (*Anopheles gambiae*) and culicine (*Aedes triseriatus*) mosquitoes (Ribeiro *et al.* 1994). Other vasoactive factors may

Correspondence: N.S. Zeidner

Received: 22 April 1998

Accepted for publication: 11 August 1998

potentiate successful mosquito feeding, as salivary gland homogenates derived from *Anopheles albimanus* oxidize noradrenalin, effectively inhibiting vasoconstrictive pathways (Ribeiro & Nussenzeig 1993). Thus, depending on the species, mosquitoes have generated very different but effective means of enhancing successful blood feeding.

Although tachykinins like substance P have numerous immune modulatory effects within the mammalian host (Siemion *et al.* 1994, Gordon *et al.* 1997), little work has been done to directly demonstrate the role of mosquito saliva and specific components of saliva in immune modulation of the mammalian host (Bissonette *et al.* 1993, Cross *et al.* 1994). Bissonette *et al.* (1993) described a factor in saliva that directly inhibits tumor necrosis factor alpha release from activated mast cells. In addition, salivary gland lysates derived from *Ae. aegypti* suppressed interleukin-2 and gamma interferon production *in vitro* and interfered with proliferation of T cells in response to interleukin-2 (Cross *et al.* 1994). The *in vivo* biological relevance of mosquito derived factors in immunosuppressing the host immune response and potentiating arborvirus transmission has not been demonstrated directly, although studies by Edwards *et al.* (1998) demonstrated the potentiation of Cache Valley Virus infection transmitted by three mosquito species as well as the potentiation of LaCrosse virus infectivity by coinoculating salivary glands from *Aedes* and *Culex* mosquitoes (Edwards J.F., personal communications). Likewise, studies by Osorio *et al.* (1996) demonstrated that *Ae. triseriatus* inoculation of LaCrosse virus led to higher titered and longer viremias in deer, as opposed to needle inoculation of virus. Similarly, salivary gland extracts derived from *Ixodid* ticks significantly enhanced the transmission of tick-borne encephalitis virus in guinea pigs (Labuda *et al.* 1993). Thus, the phenomenon of enhanced pathogenicity of infectious agents coinoculated with vector saliva appears to be conserved among arthropod vectors (Champagne 1994, Randolph & Nuttall 1994).

The studies presented here were undertaken to determine, (1) the influence of mosquito feeding on the initial T cell response in the flavivirus resistant versus flavivirus susceptible host and (2) to determine what role, if any, mosquito tachykinins like sialokinin-I and sialokinin-II have in modulating the immune response of the murine host. To our knowledge this is the first report of the direct effect of mosquito feeding on systemic T cell responses in the mammalian host and suggests multiple functions for secretion of tachykinin like molecules by mosquito vectors. This may have important ramifications for the development of alternative vaccine strategies designed to block endemic, vector-transmitted flavivirus infection.

## MATERIALS AND METHODS

### Mice

Virus free 6–8 week old C3H/HeJ mice were obtained from the Jackson Laboratory, Bar Harbor, ME, USA. Flavivirus resistant C3H/RV (C3H.PRI-FLV) mice were obtained from the Animal Resources Centre, University of Western Australia, Perth, Australia.

### Mosquito colonies and mosquito feeding of mice

Laboratory reared colonies of *Ae. aegypti* and *Cx. pipiens* were maintained as described previously (Higgs & Beaty 1996). A 'spot' feeding technique was utilized to expose mosquitoes to mice (Edwards *et al.* 1998). Briefly, individual mice were restrained within a wire mesh device. A gauze bandage with a 2 cm diameter hole exposing the dorsal skin was placed between an individual mouse and the wire mesh. Mosquitoes feeding on each mouse had access only to the 2 cm<sup>2</sup> area of exposed skin. Each mouse was then placed into a mosquito colony cage until approximately 10–25 mosquitoes had fed to repletion upon each mouse. A control, immobilized but mosquito non-exposed group of mice, were similarly treated. Three mice per group were utilized in these experiments and each experiment was repeated once ( $n = 6$ ).

### Preparation of splenocytes for cytokine production

Spleens from groups of three animals (total of six animals per time point) were harvested on days 4 through 10 after mosquito feeding or after injection of sialokinin. Individual spleens were teased apart between the frosted ends of two microscope slides and a single cell suspension of mononuclear cells was isolated by Ficoll-Hypaque gradient centrifugation (Nycoprep 1.077; Nycomed Pharma AS, Oslo, Norway). After washing three times in phosphate buffered saline (PBS), the cells were suspended at  $5 \times 10^6$  cells per ml of complete media consisting of RPMI 1640 supplemented with 10% fetal calf serum, 2% glutamine, 2% sodium bicarbonate (7.5% vol/vol), 2% essential amino acids, 1% nonessential amino acids, 1% sodium pyruvate, 100 U of penicillin per ml, 100 µg of streptomycin per ml, and  $2 \times 10^{-5}$  M 2-mercaptoethanol. A total  $5 \times 10^6$  cells were then placed in individual wells of a 24-well plate (no. 3524; Costar Corp., Cambridge, MA, USA) and stimulated with 2 µg of Concanavalin-A (Con-A) (Boehringer Mannheim, Germany) per ml. Supernatants from these cultures were harvested at either 24 h after stimulation to analyse for IL-2 or 48 h post stimulation to quantitate IFN $\gamma$ , IL-4 and IL-10 production. The supernatants were frozen at  $-70^\circ\text{C}$  until use.

### Quantification of Th1 and Th2 Cytokines after mosquito feeding

Th1 (IL-2, IFN $\gamma$ ) and Th2 (IL-4, IL-10) cytokines were quantified by antigen-capture ELISA as previously described (Zeidner *et al.* 1997) utilizing the following capture and detection monoclonal antibody pairs: IL-2, clones JES6-1A12 and JES6-5H4; IFN $\gamma$ , R464A2 and XMG1.2; IL-4, BVD4-1D11 and BVD6-24G2; and IL-10, MP6XT22 and BVD6-24G2 from Pharmingen, San Diego, CA, USA. Each sample was assayed in quadruplicate wells and the percentage of control cytokine values was determined by dividing the experimental value (pg per ml) by control values (supernatants harvested from splenocytes derived from mice that had been similarly restrained but not fed upon by mosquitoes) and multiplied by 100. Spontaneous release of cytokines from unstimulated control wells (Con-A-negative control wells) accounted for no more than 0.1% of the cytokine levels for each time point.

### Production and purification of sialokinin-I and sialokinin-II

Sialokinin-I and sialokinin-II peptides were synthesized by the Macromolecular Resource Division of the Colorado State University Bioprocessing Center based on published sequences (Champagne & Ribeiro 1994). Purity of synthesized products was confirmed by mass spectrum analysis. In addition, a guinea pig ileum isotonic contraction bioassay confirmed bioactivity of both sialokinin-I and sialokinin-II as previously described (Champagne & Ribeiro 1994).

### *In vivo* studies utilizing sialokinin-I and sialokinin-II

Groups of six mice were injected subcutaneously with 5 500 or 1000 ng of sialokinin diluted in PBS. Each mouse received a total volume of 100  $\mu$ l. Control mice ( $n=6$ ) were inoculated with 100  $\mu$ l of PBS and splenocytes were harvested from mice at 4, 7, or 10 days after inoculation with sialokinin and cultured for cytokine production as described earlier.

### Statistical analysis

Significant differences in the mean levels of cytokine production were determined by Student's *t*-test. *P* values less than 0.05 were considered statistically significant.

## RESULTS

### Systemic cytokine production after mosquito feeding on C3H/HeJ mice

In order to determine the ability of splenocytes to produce

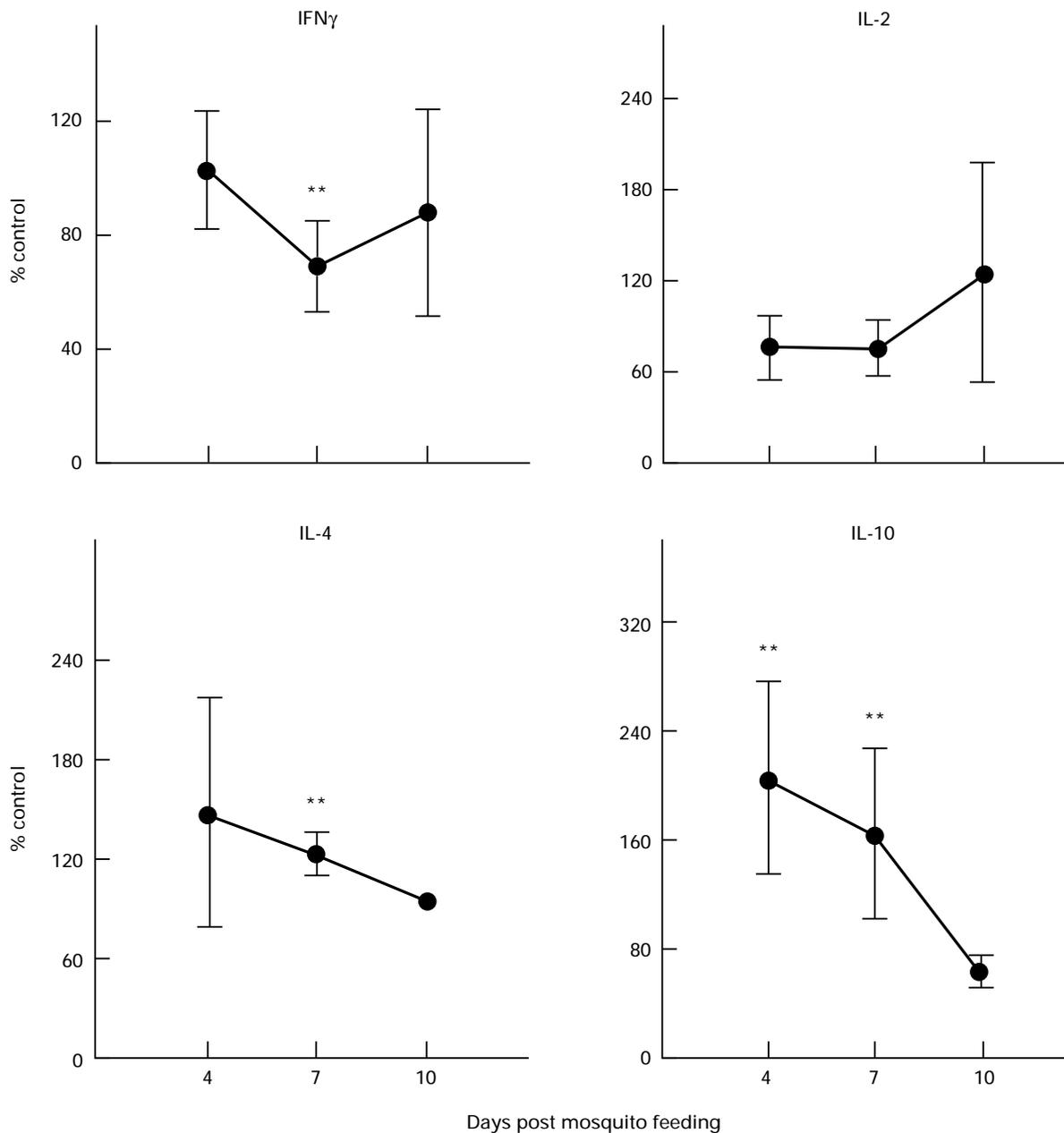
both Th1 and Th2 cytokines after *Ae. aegypti* feeding, C3H/HeJ mice ( $n=6$ /time point) were restrained in wire cages and exposed to adult mosquitoes for one h. On average, a total of ten mosquitoes fed on restrained mice, and mice were sacrificed at days 4–10 in order to quantitate the ability of splenocytes to produce Th1 and Th2 cytokines. IFN $\gamma$  production was significantly decreased by 28% of control values at day 7 ( $P<0.0002$ ), while Th2 cytokines IL-4 and IL-10 were significantly up regulated (120% and 160–225% of control values) at days 7 and 10 ( $P<0.0006$ ,  $P<0.004$ ) (Figure 1). Likewise, feeding of *Cx. pipiens* mosquitoes on C3H/HeJ mice (Figure 2) significantly suppressed IFN $\gamma$  production from days 4 through 10, ranging from 38% of control on day 4 and recovering to only 65% of control values by day 10 ( $P<0.002$ ). Likewise, Th2 cytokine production was significantly up regulated. IL-4 values were 160% ( $P=0.05$ ) of control at day 10 after *Cx. pipiens* feeding, while *Ae. aegypti* feeding induced significant up regulation of IL-10 at days 4 (200% of control values,  $P<0.004$ ) and 7 (165% of control values,  $P<0.02$ ) concurrent with a significant up regulation of IL-4 (120% of control values,  $P<0.006$ ) occurring at day 7 after mosquito feeding. As was noted in the case of *Ae. aegypti* feeding, IL-2 production was not significantly different between mice fed upon by mosquitoes and mock-fed control animals.

### Systemic cytokine production after *Cx. pipiens* feeding on C3H/RV mice

No significant difference was noted in the number of mosquitoes (av. = 10/mouse) that fed upon flavivirus-resistant animals compared to C3H/HeJ mice (data not shown). In terms of cytokine production after mosquito feeding on congenic C3H/RV mice, IFN $\gamma$  was significantly up regulated ten days after mosquito feeding (125.6 ng/ml,  $P<0.027$ ) compared to mock-fed C3H/RV mice (Figure 3). The total amount of IFN $\gamma$  stimulated, in the range of 95–150 000 pg/ml, was greater than that demonstrated for C3H/HeJ mice, which ranged between 7–37 000 pg/ml. Likewise, IL-2 production was up regulated (7975 pg/ml,  $P=0.003$ ) seven days after mosquito feeding. In contrast to C3H/HeJ mice, IL-4 production was significantly decreased in C3H/RV mice ten days after *Cx. pipiens* feeding ( $P<0.006$ ) compared to mock-fed control animals, while IL-10 was significantly elevated at days seven and ten (120–130 pg/ml,  $P=0.02$ ).

### Systemic cytokine production after subcutaneous injection of sialokinin-I and sialokinin-II in C3H/HeJ mice

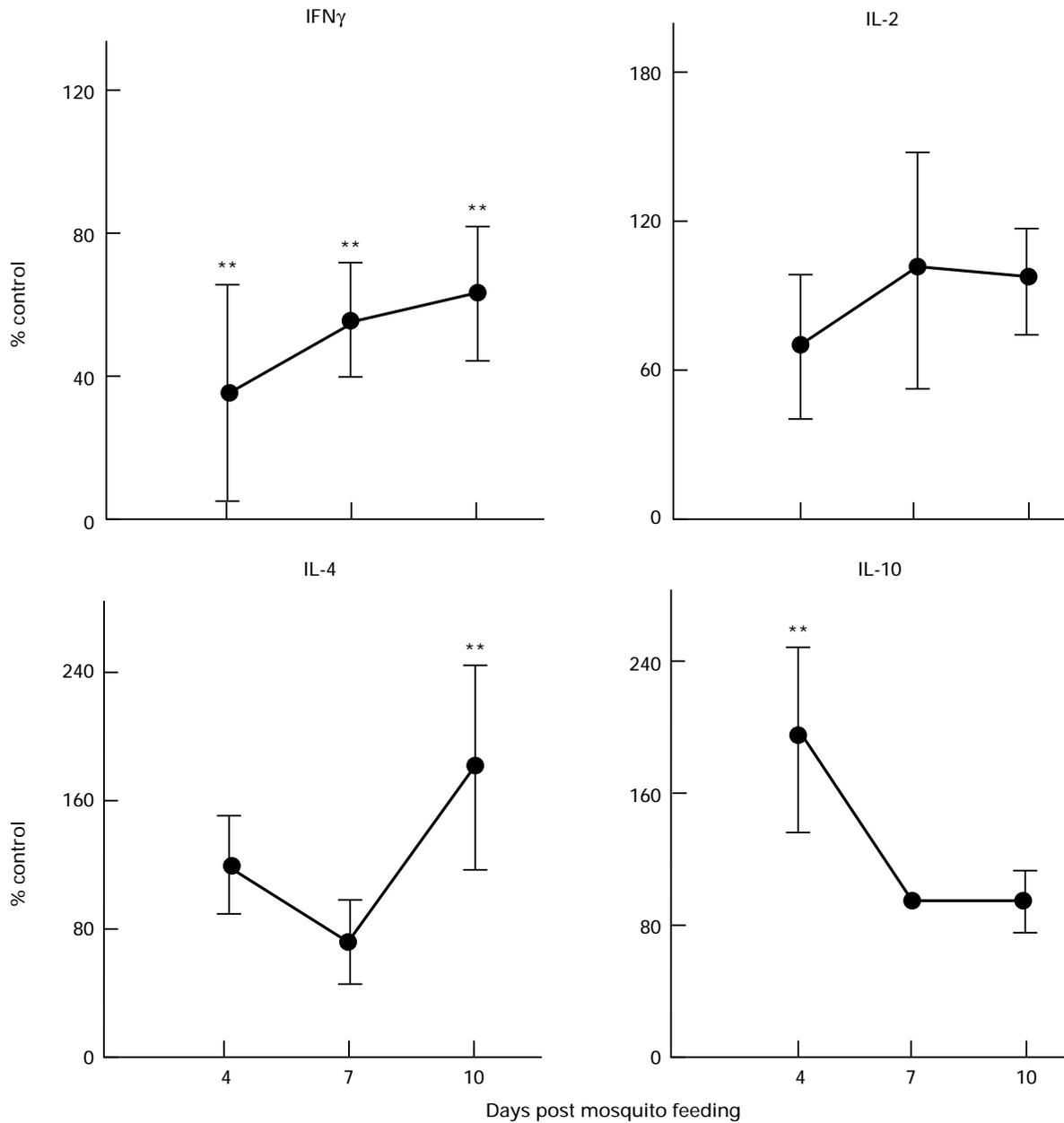
To determine whether *Ae. aegypti* vasodilatory peptides,



**Figure 1** Th1 (IFN $\gamma$  and IL-2)-associated and Th2 (IL-4 and IL-10)-associated cytokine production by splenocytes harvested from C3H/HeJ mice after *Ae. aegypti* feeding. Splenocytes were harvested from individual mice on the days indicated and simulated with Con-A. Cytokine levels in supernatants were then quantitated by antigen capture ELISA. Per cent control is equal to the cytokine level (pg per ml) in the experimental group divided by levels obtained from naive mice (restrained, but unexposed) and multiplied by 100. \*\* indicates statistically significant ( $P < 0.05$ ) differences between the experimental and the unexposed group.  $n = 6$  animals per time point. Bars represent standard deviation (SD) from the mean.

sialokinin-I or sialokinin-II modulated systemic cytokine production, each peptide was injected separately into groups of mice ( $n = 6$ ), and cytokine production was subsequently quantified on days 4–10 and compared to mice injected with the diluent, PBS. As noted in Table 1, sialokinin-I

profoundly effected both Th1 and Th2 cytokines in C3H/HeJ mice. In terms of Th1 cytokine production, both IFN $\gamma$  and IL-2 were significantly down regulated four days after injection of sialokinin-I (Table 1). IFN $\gamma$  values ranged from 49 to 63% of control values, depending on the concentration

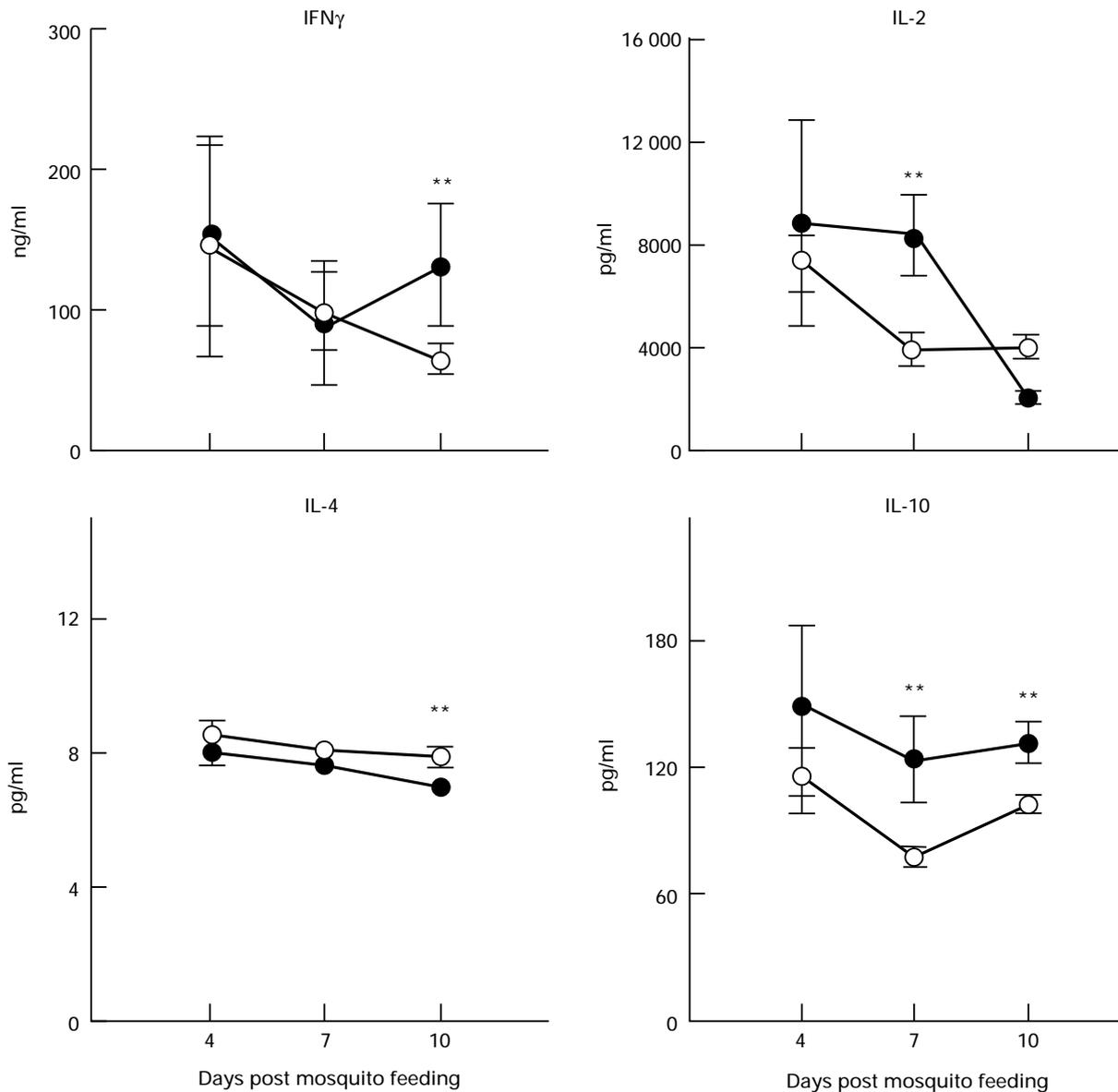


**Figure 2** Th1 (IFN $\gamma$  and IL-2)-associated and Th2 (IL-4 and IL-10)-associated cytokine production by splenocytes harvested from C3H/HeJ mice after *Cx. pipiens* feeding. Splenocytes were harvested from individual mice on the days indicated and stimulated with Con-A. Cytokine levels in supernatants were then quantitated by antigen capture ELISA. Per cent control is equal to the cytokine level (pg per ml) in the experimental group divided by levels obtained from naive mice (restrained, but unexposed) and multiplied by 100. \*\* indicates statistically significant ( $P < 0.05$ ) differences between the experimental and the unexposed group.  $n = 6$  animals per time point. Bars represent SD from mean.

of sialokinin-I injected (Table 1). IL-2 production was profoundly suppressed in animals injected with 5 ng of sialokinin-I (36% of control,  $P = 0.00003$ ). Moreover, Th2 cytokines, IL-4 and IL-10, were significantly up regulated in animals injected with 5 ng of sialokinin-I (Table 1). As in the case of IL-2, greater up regulation of these cytokines was

noted in animals injected with the lowest dose of sialokinin-I (5 ng). Except for IL-2, in which production remained suppressed over a seven day period (59–60% of PBS control values), IFN $\gamma$ , IL-4 and IL-10 rebounded to normal control levels by day 10 after injection.

When C3H/HeJ mice were exposed to sialokinin-II, only



**Figure 3** Th1 (IFN $\gamma$  and IL-2)-associated and Th2 (IL-4 and IL-10)-associated cytokine production by splenocytes harvested from C3H/RV mice after *Cx. pipiens* feeding. Splenocytes were harvested from individual mice on the days indicated and stimulated with Con-A. Cytokine levels in supernatants were then quantitated by antigen capture ELISA. Mock-fed animals refers to mice that were restrained, but not fed upon by mosquitoes. \*\* indicates statistically significant ( $P < 0.05$ ) differences between the experimental and the mock-fed group.  $n = 3$  animals per time point. ● mosquito-fed; ○ mock-fed. Bars represent SD from the mean.

minimal systemic effects on splenic cytokine production were noted (Table 2). Splenic IFN $\gamma$  production was significantly suppressed ten days after injection (66–78% of control) when utilizing higher concentrations sialokinin-II. Likewise, in terms of Th2 cytokines, only IL-4 was significantly up regulated, between 179 and 284% of control values, which occurred at day 4–7 after injection of 5 ng of sialokinin-II (Table 2).

## DISCUSSION

The studies presented here indicate that the process of mosquito feeding had a significant impact on the ability of murine splenocytes to produce both Th1 and Th2 derived cytokines. Natural feeding by both *Ae. aegypti* and *Cx. pipiens* significantly down regulated IFN $\gamma$ , while up regulating Th2 cytokines, IL-4 and IL-10. Natural feeding by

**Table 1** Effect of sialokinin-I on cytokine production in C3H/HeJ mice

		Day 4*	Day 7	Day 10
IFN $\gamma$	5 ng**	<b>62.6 <math>\pm</math> 17***</b>	87.4 $\pm$ 15	93 $\pm$ 20
	500 ng	<b>49.1 <math>\pm</math> 25</b>	69.4 $\pm$ 20	85.9 $\pm$ 18
	1000 ng	<b>53.9 <math>\pm</math> 15</b>	82.9 $\pm$ 1.1	70.4 $\pm$ 7
IL-2	5 ng	<b>35.7 <math>\pm</math> 9</b>	<b>60.0 <math>\pm</math> 9.4</b>	86.3 $\pm$ 15
	500 ng	<b>57.9 <math>\pm</math> 14</b>	<b>59.8 <math>\pm</math> 10</b>	92.9 $\pm$ 15
	1000 ng	<b>76.4 <math>\pm</math> 11</b>	<b>58.5 <math>\pm</math> 12</b>	110.9 $\pm$ 2.3
IL-4	5 ng	<b>860 <math>\pm</math> 73</b>	96.8 $\pm$ 9	90.1 $\pm$ 20
	500 ng	<b>596 <math>\pm</math> 391</b>	100.7 $\pm$ 8.1	119.3 $\pm$ 20
	1000 ng	<b>542 <math>\pm</math> 261</b>	49.3 $\pm$ 38	116.8 $\pm$ 10
IL-10	5 ng	<b>239 <math>\pm</math> 120</b>	76.5 $\pm$ 46	64.8 $\pm$ 30
	500 ng	<b>151.0 <math>\pm</math> 61</b>	84.4 $\pm$ 46	103.3 $\pm$ 6.9
	1000 ng	<b>121.6 <math>\pm</math> 51</b>	93.2 $\pm$ 28	109.5 $\pm$ 7.3

Splenocytes were harvested at the days indicated post injection of sialokinin-I and stimulated with Con-A. Cytokine levels in supernatants were then quantitated by antigen capture ELISA. Per cent control is equal to the cytokine level (pg per ml) in the experimental group divided by levels obtained from animals inoculated with PBS and multiplied by 100. Numbers in **bold** type indicate statistically significant ( $P < 0.05$ ) differences between the experimental and control group which received PBS.  $n = 6$  animals per time point.

\*days post inoculation of sialokinin-I; \*\*concentration of sialokinin-I inoculation; \*\*\*percent control of cytokine measured.

both vectors did not influence the ability of splenocytes to produce IL-2 after mitogen stimulation. These results contrast with those reported by Cross *et al.* (1994), which indicated that salivary gland extract derived from *Ac. aegypti*, suppressed both Con-A driven lymphocyte proliferation as well as IL-2 production *in vitro*. In these studies, up to 25 ng/ml of salivary gland extract was used *in vitro* to induce this suppressive effect. This indicated that factors produced in saliva are released during blood feeding at lower concentrations that may suppress IFN $\gamma$  production, but not effect IL-2 release in response to mitogenic stimulation. Similarly, in contrast to Cross *et al.* (1994), we demonstrated that Th2 cytokines, IL-4 and IL-10, are significantly up regulated four to ten days after mosquito feeding, depending on the vector studied. Again, whether the amount of salivary gland lysate utilized in those studies by Cross *et al.* (1994) did not accurately reflect molecules delivered to the host by natural feeding, or whether other unique molecules are being produced and released by salivary glands during the feeding process remains to be

**Table 2** Effect of sialokinin-II on cytokine production in C3H/HeJ mice

		Day 4*	Day 7	Day 10
IFN $\gamma$	5 ng	106.8 $\pm$ 13***	96.7 $\pm$ 18	81 $\pm$ 33
	500 ng	83.3 $\pm$ 18.4	104 $\pm$ 15	<b>65.6 <math>\pm</math> 17</b>
	1000 ng	98.7 $\pm$ 16	103 $\pm$ 50	<b>77.7 <math>\pm</math> 16</b>
IL2	5 ng	91.5 $\pm$ 5.3	92.6 $\pm$ 3.9	115 $\pm$ 50
	500 ng	97.2 $\pm$ 7.2	112 $\pm$ 14	84 $\pm$ 27
	1000 ng	158.5 $\pm$ 86	95.4 $\pm$ 17	133 $\pm$ 49
IL-4	5 ng	<b>277 <math>\pm</math> 57</b>	<b>284 <math>\pm</math> 20</b>	72 $\pm$ 18
	500 ng	190 $\pm$ 96	<b>179 <math>\pm</math> 39</b>	52.4 $\pm$ 15
	1000 ng	223 $\pm$ 103	<b>191 <math>\pm</math> 32</b>	53.4 $\pm$ 13
IL-10	5 ng	ND	108.9 $\pm$ 10	80.4 $\pm$ 10
	500 ng	ND	100.9 $\pm$ 7	105.7 $\pm$ 4
	1000 ng	ND	89.7 $\pm$ 6	87.6 $\pm$ 5

Splenocytes were harvested at the days indicated post injection of sialokinin-II and stimulated with Con-A. Cytokine levels in supernatants were then quantitated by antigen capture ELISA. Per cent control is equal to the cytokine level (pg per ml) in the experimental group divided by levels obtained from animals inoculated with PBS and multiplied by 100. Numbers in **bold** type indicate statistically significant ( $P < 0.05$ ) differences between the experimental and control group which received PBS.  $n = 6$  animals per time point.

\*days post inoculation of sialokinin-II; \*\*concentration of sialokinin-II inoculated; \*\*\*percent control of cytokine measured.

determined. Previous studies indicated that the complexity of gene expression and resultant salivary gland products is temporally modified with maturation of the mosquito as well as in response to blood feeding (Orr *et al.* 1961, James 1994). These studies demonstrate the need for a natural feeding model to study the role of vectors on modulating the host immune response (Randolph & Nuttall 1994).

In contrast to what was seen in flavivirus susceptible (C3H/HeJ) mice, mosquito feeding on congenic, flavivirus resistant mice (C3H/RV) produced opposite results. Our data indicate that *Cx. pipiens* feeding significantly up regulated Th 1 cytokines, IFN $\gamma$  and IL-2, while significantly down regulating IL-4. Only IL-10 was significantly up regulated at days 7 through to 10 post feeding, which mirrored, to some extent, feeding by *Cx. pipiens* on susceptible mice. To our knowledge, this is the first study investigating the effects of natural vector feeding on C3H/RV mice. Previous studies indicated that genetic differences between C3H/RV and C3H/HeJ mice map to two loci on chromosome 5 (Sangster *et al.* 1993). These changes, which

induced lower flavivirus titres both *in vitro* and *in vivo*, resulted in reduced production of replicative forms of viral RNA as well as the production of more efficient defective viral particles in resistant mice (Vainio *et al.* 1961, Darnell *et al.* 1974, Brinton 1983). In terms of studying the effect of these changes on the immune response to viral infection, data from Brinton *et al.* indicated that resistance to yellow fever virus inoculation in resistant mice might be independent of interferon production (Brinton *et al.* 1982). However, these studies utilized syringe inoculation of yellow fever virus and only examined the effect of neutralization of alpha and beta interferon on viral titers within the central nervous system (Brinton *et al.* 1982). Our studies and those of Bhatt & Jacoby (1976) indicate that multiple immune regulatory factors may be playing a role in flavivirus resistance *in vivo* within this genetically resistant strain of mice.

The studies presented here indicate that the early suppression of IFN $\gamma$  and possibly the up regulation of IL-4, may be significant events in the early escape of immune surveillance by flavivirus introduced by feeding vectors in susceptible animals. The significance of these observations are as follows. *In vitro* studies have indicated that cytotoxic T lymphocytes block the release of Japanese encephalitis (JE) virus from infected Neuro 2a and P388D1 cells (Murali-Krishna *et al.* 1994), and recent data from Lin *et al.* (1997) indicate that inhibition of replication of JE virus in an IFN $\gamma$  activated murine macrophage cell line was correlated to the production of nitric oxide in these cells. Likewise, *in vivo*, cytotoxic CD4<sup>+</sup> T cells can be utilized in adoptive transfer experiments to protect adult mice from intracerebral challenge with JE virus (Murali-Krishna *et al.* 1996). These experiments illustrate the relative importance of IFN $\gamma$  in inducing direct cellular cytotoxicity against virus and/or the release of mediators of virus inhibition by activated antigen presenting cells. Natural killer (NK) cells have been shown to be an early source of IFN $\gamma$  when stimulated with IL-2 in the presence of accessory cells, and IFN $\gamma$  produced by NK cells enhances the ability of antigen-presenting cells (macrophages) to shift the T helper response to a Th1 type response (Trinchieri 1989, 1995). Given that the early source of IFN $\gamma$  *in vivo* may be NK cells circulating throughout the skin (Trinchieri 1989) and that NK cell activity may be blocked by molecules secreted by other arthropod vectors to enhance viral infection *in vivo* (Jones *et al.* 1992, Kubes *et al.* 1994, Kopeccky 1995), it is likely that similar phenomena may enhance mosquito transmitted virus infection *in vivo*. Studies conducted with other arthropod vectors, like ticks, indicate that vector saliva may directly suppress NK cell cytotoxicity as well as IFN $\gamma$  release *in vitro* (Kubes *et al.* 1994, Kopeccky 1995). Whether mosquito feeding also down regulates effector cells and antigen

presentation within the skin, thereby enhancing pathogenicity of flavivirus infection within the mammalian host, needs to be determined.

The role for sialokinins in producing the *in vivo* modulation of Th1 and Th2 cytokines was also investigated. Injection of sialokinin-I into flavivirus susceptible mice had a profound impact on the ability of splenocytes to produce both IFN $\gamma$  and IL-2. IFN $\gamma$  production was significantly suppressed four days after inoculation, while IL-2 was suppressed throughout a seven-day period after injection. Concomitantly, both IL-4 and IL-10 were significantly up regulated four days after injection of sialokinin-I and mirrored what was detected 4–7 days after *Ae. aegypti* feeding. Previous reports have indicated that tachykinins, like mammalian substance P, can activate mononuclear cells and greatly influence the local cytokine milieu (Hartung & Toyka 1983, Foreman 1987, Pascual & Bost 1990, Gordon *et al.* 1997). Studies by Pascual *et al.* (1990) and Hartung *et al.* (1983) indicated that substance P can activate macrophage cytotoxicity by inducing superoxide radical formation and the release of IL-1. Likewise, this neuropeptide has been shown to induce mast cells to produce tumor necrosis factor (Ansel *et al.* 1993), augment IL-10 production by macrophages (Ho *et al.* 1996), and modulate both the production of IFN $\gamma$  and IL-4 by human lymphoid cells. This leads to a preferential Th2 response and enhanced disease in patients with atopic dermatitis (Gordon *et al.* 1997). Our studies indicate that in addition to their role as vasodilators during mosquito feeding, sialokinins significantly impact the systemic T cell immune response after vector feeding in the flavivirus susceptible host. Given the fact that IL-2 is down regulated by inoculation of sialokinin-I and that feeding by both *Ae. aegypti* and *Cx. pipiens* had no significant effect on IL-2 production, it is evident that other immune regulatory molecules are released in mosquito saliva to mediate effects on cytokine release. Alternatively, the studies presented here did not adequately mimic the concentration of sialokinin-I release *in vivo*. In either case, our studies indicate at least a dual role for release of sialokinin-I and sialokinin-II by mosquito vectors. These neuropeptides induce vasodilation to facilitate the cannulation of microvasculature and modulate the host T cell immune response, polarizing that response toward a Th2 response 4 to 7 days after feeding. In an evolutionary sense, sialokinins released in saliva promote successful vector feeding and may well enhance survival and pathogenicity of virus transmission in a compromised mammalian host.

Polarization of the host immune response to a predominantly Th2 response by lymphocytes after mosquito feeding may have a significant impact on mosquito transmission of viral pathogens like flaviviruses. Modulation of the host immune response away from factors like IFN $\gamma$  that promote

both innate immunity in the form of NK cell immune surveillance and antigen presentation, may provide a significant window of opportunity for virus transmission to target organs of the mammalian host. Recent experimental data has indicated that either exogenous or endogenous reconstitution of Th1 cytokines at the time of arthropod vector feeding restored innate immunity and blocked tick transmitted bacterial infection in the murine host (Zeidner *et al.* 1996). To that end, experiments are underway to determine whether similar strategies can reverse the immune modulatory effect of mosquito feeding *in vivo* and block mosquito transmitted flavivirus infection in the genetically susceptible mammalian host.

#### ACKNOWLEDGEMENTS

The authors wish to thank Cynthia Oray, Marc C. Dolan and Edwin Baca for their expert technical assistance and Dr Jose M.C. Ribeiro for providing the bioassay to test synthetic peptides sialokinin-I and sialokinin-II. The authors would also like to thank Dr Donald Champagne for helpful discussions in designing experimental procedures.

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