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Kinetics of ingested host immunoglobulin G in hemolymph and whole body homogenates during nymphal development of *Dermacentor variabilis* and *Ixodes scapularis* ticks (Acari: Ixodidae).

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Abstract. Patterns in the utilization of host immunoglobulin G (IgG) during nymphal development differed between *Dermacentor varibilis* (Say) and *Ixodes scapularis Say* ticks. In unfed nymphs of *D. variabilis*, host IgG was readily detectable in both hemolymph and whole body homogenates. In unfed nymphs of *I. scapularis*, host IgG was absent in hemolymph and at very low concentrations in whole body homogenates. Host IgG in unfed nymphs was undoubtedly the remnants of IgG acquired during the larval bloodmeal that persisted through metamorphosis to the nymphal stage. In both tick species, host IgG crossed the midgut into the hemocoel during the latter phases of engorgement. Concentrations of host IgG in *I. scapularis* declined considerably after replete nymphs molted to the adult stage. In contrast, concentrations of host IgG in *D. variabilis* remained elevated throughout metamorphosis to the adult stage. When larval *D. variabilis* were fed on a rat, then 2 months later as nymphs on a rabbit, the rat IgG ("old IgG") present in unfed nymphs was totally replaced by rabbit IgG ("new IgG") within 2 d of nymphs attaching to the rabbit. Presumably, the old IgG acquired from a previous bloodmeal was secreted via saliva into the new host.

Introduction

Ixodid ticks obtain nutrients solely from vertebrate host blood. An important constituent of vertebrate blood is antibody, particularly immunoglobulin G (IgG). The obligatory ingestion of host IgG by ticks can be exploited in at least 2 ways. First, host animals immunized against tick components (e.g., midgut proteins) may reduce the survival or fecundity of ticks (Chinzei and Minoura 1988; da Silva Vaz et al. 1998; de la Fuente et al. 1998; Fragoso et al. 1998; Kimaro and Opdebeeck 1994; McKenna et al. 1998; Rodriguez et al. 1995; Willadsen et al. 1993, 1995). Second, ingested IgG directed against tick-borne pathogens may inhibit or block

pathogen development within the tick vector (Azad et al. 1989; Fujisaki et al. 1984). Thus, increased knowledge on the way in which ticks process ingested host IgG can contribute to the development of new strategies against ticks and tick-borne diseases.

A salient feature of ixodid ticks is the passage of a small amount of ingested IgG from the bloodmeal into the hemocoel (Ackerman et al. 1981; Ben-Yakir et al. 1987; Ben-Yakir 1989; Brossard and Rais 1984; da Silva Vaz et al. 1998; Fujisaki et al. 1984; Tracey-Patte et al. 1987). The fact that IgG passage is such a widespread phenomenon among ixodid ticks suggests that IgG passage has some fundamental importance in their overall physiology. The precise manner by which IgG passes through the tick midgut is not known but recent studies (Jasinskas et al. 2000) suggest that ticks may possess a specific mechanism for the selective uptake and passage of host IgG into the hemocoel. Indeed, ticks are apparently able to regulate the host IgG within the hemocoel. Immunoglobulins in the hemocoel are "scavenged" by immunoglobulin-binding proteins present within tick hemolymph and transported to another set of immunoglobulin-binding proteins in tick salivary glands. The IgGs are then excreted by the salivary glands in the tick's saliva (Wang and Nuttall 1994, 1995a, 1995b).

Studies on the kinetics of IgG processing in ticks have been conducted exclusively with adult ticks. There are no published accounts defining the extent or kinetics of IgG passage in immature ixodid ticks. The objective of this study is to compare the persistence and utilization of host IgG during nymphal development of the American dog tick, *Dermacentor varibilis* (a metastriate tick), and the black-legged tick, *Ixodes scapularis* (a prostriate tick).

Materials and methods

Tick feeding

All *D. variabilis* and *I. scapularis* ticks used in these studies were derived from laboratory colonies maintained at Old Dominion University, Norfolk, Virginia, USA. Colony progenitors were screened and certified free of spotted fever group rickettisae *(D. variabilis)* and spirochetes *(I. scapularis)*. Three experimental host infestations were conducted using established techniques (Sonenshine 1991). The first host infestation examined the general pattern of host IgG utilization during *D. variabilis* nymphal feeding and development. Approximately 100 nymphal *D. variabilis* were released onto the body of a female Sprague-Dawley rat. The rat was lightly restrained in hardware cloth for 1 h in order to prevent host grooming and allow the ticks to attach. The infested rat was subsequently housed in a wire-bottomed cage suspended over a tray supplied with several layers of paper towels. This facilitated the containment and collection of replete ticks that dropped off the rat 5 to 7 days later. The second host infestation examined the general pattern of host IgG utilizafion during nymphal *I. scapularis* feeding and development. Approxi-

mately 100 *I. scapularis* nymphs were placed in a specialized feeding capsule taped to the side of a female New Zealand rabbit (Sonenshine 1993). The Plexiglass feeding capsule was constructed with a screw top, allowing access to the ticks during the week-long feeding process. The third host infestation examined the pattern of host-specific IgG utilization in *D. variabilis* when a cohort of ticks was first fed on a rat as larvae, then on a rabbit as nymphs. All experimental host infestations were conducted under the guidelines of a protocol approved by our Institutional Animal Care and Use Committees.

Sample Collection

Tick hemolymph and whole body homogenates were collected from individual ticks and assayed for host IgG at various stages throughout nymphal development. The stages examined were; unfed nymphs 6 to 8 weeks after molting (=flat nymphs), 2 days after host attachment, 3 days after host attachment, 4 days after host attachment, 5 days after dropping off the host (=replete nymphs), and 2 to 7 days after molting to the adult stage (=flat adult). Ticks were vortexed twice in phosphate buffered saline plus 0.5% Tween (PBS-TW) and once in PBS to remove any blood residue or tick feces on their surfaces. Ticks were immobilized by chilling and further manipulations were conducted with the aid of a dissecting microscope and chill table. Hemolymph samples were collected and the bodies were then homogenized in individual glass micro-grinders. In the first experiment, hemolymph was collected from *D. variabilis* ticks by amputating one or more legs. Exuding hemolymph was collected into tapered microcapillary tubes containing a measured amount of casein blocking buffer. In the subsequent experiments, hemolymph was collected using an improved technique that allowed a greater proporuon of the total hemolymph content of an individual tick to be collected — namely, hemocoel perfusion. Ticks were placed ventrally in a small (30 μl) drop of blocking buffer and a tiny section of the posterior marginal body fold was excised, exposing the tick hemocoel. A fine-tipped glass needle, formed from a 10 μl microcapillary, was connected via tubing to a large (500 ml) syringe secured to a ring-stand. The needle was filled with ca 7 μl of blocking buffer and inserted into the foramen formed at the juncture of the capitulum and scutum (dorsal shield). Applying gentle pressure to the plunger of the syringe, tick hemolyrnph, ranging in color from amber (flat nymphs and adults) to virtually clear (replete nymphs), was flushed out of the hemocoel and into the drop of blocking buffer. With replete ticks, the technique had to be modified such that ticks were first decapitated, the needle inserted at a diagonal to the long axis of the tick body and the perfusate expressed from the anterior foramen. To safeguard against possible host blood meal contamination, only clear hemolymph was used in antibody assays. Hemolymph and whole body homogenates were labeled, kept on ice during sample collection and subsequently stored at −70 °C until assayed.

Immunoassays

Total host IgG in tick hemolymph and whole body homogenates was detected with an antibody-capture enzyme-linked immunosorbent assay (ELISA), using affinity-purified goat anti-rat or anti-rabbit IgG (H+L) as the capture antibody. In the third experiment, it was imperative to be able to distinguish between rat IgG and rabbit IgG. Therefore, ELISAs for the third experiment utilized affinity-purified capture antibody specifically adsorbed against the serum proteins of the heterologous species (Kirkegaard & Perry, Gaithersburg, Maryland USA). Preliminary ELISAs using reagent grade rat and rabbit lgGs confirmed the manufacturer's claim of non-cross-reactivity (data not shown). Optimal concentrations for capture antibodies were determined in preliminary checkerboard titrations. Fifty μl; of capture antibody solution in PBS were added to each well of polystyrene microtiter plates and incubated overnight at 4 °C. Wells were emptied and 150 μl of casein blocking buffer (Vaughan et al. 1998) were added to each well. After a 1 h blocking step, wells were aspirated and washed 3 times with PBS-TW. Test samples of tick hemolymph and whole body homogenates were diluted in casein buffer and added to the wells. Each plate also included wells containing casein buffer only (blanks) and reagent-grade host IgG diluted in buffer (standard curve). Serial 3-fold dilutions were performed using casein buffer and samples were incubated (@ 2 h, RT). Wells were aspirated and washed 3 times with PBS-TW. Peroxidase-conjugated goat anti-rat or anti-rabbit IgG (H+L) was diluted 1:500 in casein buffer and 50 μl was added to each well. After 1 h incubation, wells were aspirated, washed 3 times with PBS-TW and TMB component 1 (Kirkegaard & Perry, Gaithersburg, Maryland USA) was added to each well. Plates were observed and when blue color had developed sufficiently in the standard curve wells, TMB Component 2 was added to stop the colorimetic reaction and enhance assay sensitivity. Absorbance values (450 nm) were recorded using an ELISA plate reader (Dynatech Lab., McLean, Virginia USA). The corrected absorbance for each sample was calculated as the raw absorbance minus the mean absorbance for blanks. The estimated quantity of host IgG in a sample was calculated from the equation of the linear portion of the standard curve and multiplied by its dilution factor. It should be noted that immunoassays detected the total IgG within a sample but did not distinguish between intact IgG molecules and IgG fragments.

Results

The kinetics of host IgG utilization during nymphal development differed between *D. variabilis* and *I. scapularis* ticks. In *D. variabilis*, host IgG was present in the hemolymph (49 ng IgG) of flat nymphs (Table 1). Presumably, this represented host IgG that was acquired during the previous larval bloodmeal and persisted through the larval molt. During the early phase of nymphal attachment to the host rat, IgG concentrations in the hemolymph declined from 7 ng/sample on Day 2 to 1

ng/sample on Day 3 after host attachment. On Day 4 of host attachment, nymphal ticks began the rapid feeding phase (i.e., the "big sip") and host IgG content in the hemolymph began to increase (13 ng/sample). Concentrations of hemolymph IgG in nymphal *D. variabilis* were elevated after ticks dropped off the host (16 ng/sample) and remained elevated even after ticks had molted to the adult stage (21 ng/sample). Host IgG was present in the whole body homogenates of unfed *D. variabilis* nymphs ($\leqslant$ 6,000 ng/sample) and IgG concentrations rose steadily during nymphal engorgement and intake of host blood. Somewhat surprisingly, the concentrations of host IgG in whole body homogenates remained elevated throughout tick metamorphosis to the adult stage (87,720 ng/sample). Presumably, most of the IgG content in whole body homogenates was contained in the tick digestive tract.

In unfed nymphs of *I. scapularis*, host IgG was absent in the hemolymph (Table 2). Concentrations of host IgG in the hemolymph of *I. scapularis* nymphs peaked during the late phase of host attachment on Day 4 (78 ng/sample), but began to decline after ticks had dropped off the host (45 ng/sample) and was nearly absent after ticks had molted to the adult stage (2 ng/sample). A similar pattern was observed for whole body homogenates. Concentrations of host IgG were comparatively low in whole body homogenates of unfed *I. scapularis* nymphs (24 ± 24 ng/sample) but IgG concentrations increased during Days 3 and 4 of host attachment (18,210 ± 4,681 ng/sample and 82,572 ± 4,464 ng/sample, respectively). Concentrations of host IgG in whole body homogenates of replete *I. scapularis* nymphs were very high (277,504 ng/sample)—over three times greater than measured in replete *D. variabilis* nymphs (Table 1). However, these very high levels of host IgG declined over 300-fold (756 ng/sample) after replete *I. scapularis* nymphs had molted to the adult stage.

The finding that host IgG was present in unfed nymphs of *D. variabilis* prompted us to wonder what happened to this IgG (presumably acquired during the larval blood meal) upon the influx of new host IgG acquired during the nymphal blood meal. To investigate this, we fed larval *D. variabilis* on a rat, then 2 months later fed the resulting nymphs on a rabbit. Hemolymph and whole body homogenates were assayed for both rat and rabbit IgG. The larvally-acquired rat IgG (i.e, the "old" IgG) in the hemolymph and whole bodies of unfed nymphs were totally replaced by rabbit IgG (i.e., the "new" IgG) within 2 days of nymphs attaching to the rabbit. Ticks contained either rat or rabbit IgG but never both (Table 3), suggesting that most, if not all, of the "old" rat IgG was voided or otherwise eliminated by feeding ticks prior to the intake of "new" rabbit IgG.

Discussion

The 2 tick species examined in this study, *D. variabilis* and *I. scapularis*, displayed different patterns of host IgG processing during nymphal development. Both species exhibited rapid influx of host IgG into the body, as well as passage into the

Table 1. Demacentor variabilis. Concentration (ng/sample) of rat immunoglobulin G in hemolymph (=excised legs) and whole body homogenates during nymphal tick development.

	Flat nymphs	Nymphs Put on Host	Nymphs Attached to Host			Nymphs Drop off Host	Engorged Nymphs	Molt	Flat Adults
	-30 d	Day 0	+2 d	+3 d	+4 d	+5 to 7 d	+12 d	+35 d	+36 to 38 d
Hemolymphs	48.8±7.9		6.9±4.8	0.9±0.8	12.6±10.7		16.4±7.6		21.0±4.3
Body	⩽ 6,000		52,920	74,112	85,376		88,663		87,720
			±1,439	±2,693	±1,305		±716		±1,131
No. Ticks	10		8	10	10		7		8

Table 2. Ixodes scapularis. Concentration (ng/sample) of rabbit immunoglobulin G in hemolymph (=hemocoel perfusates) and whole body homogenates during nymphal tick development.

	Flat nymphs	Nymphs Put on Host	Nymphs Attached to Host		Nymphs Drop off Host	Engorged Nymphs	Molt	Flat Adults
	-30 d	Day 0	+3 d	+4 d	+5 to 7 d	+10 d	-	+34 to 38 d
Hemolymph	0.0		15.2±5.0	77.8±7.9		45.1±11.6		2.1±1.0
Body	24±24		18,210 ±4,681	82,572 ±4,464		277,504 ±61,558		756 ±476
No. Ticks	2		36	32		10		18

Table 3. Dermacentor variabilis. Rapid replacement of larvally-acquired rat IgG with rabbit IgG during nymphal tick feeding. Larval ticks were fed on a rat and allowed to molt to the nymphal stage. Two months later, nymphal ticks were fed on a rabbit.

Tick lifestage and tissue assayed	α-Rat IgG (ng/sample)	α-Rabbit IgG (ng/sample)
Flat nymphs fed as a larvae 2 months earlier on rat		
Hemolymph	6.9±4.8 (n=5)	0 (n=5)
Body homogenate	30,904±22,445 (n=5)	0 (n=5)
2 days after nymphal attachment to rabbit		
Hemolymph	0 (n=5)	65.9±6.4 (n=4)
Body homogenate	0 (n=5)	61,300±2,194 (n=4)
3 days after nymphal attachment to rabbit		
Hemolymph	0 (n=5)	4.8±2.9 (n=5)
Body homogenate	0 (n=5)	83,832±2,639 (n=5)

hemolymph, during engorgement. However, *I. scapularis* exhibited relatively rapid clearance of ingested host IgG so that comparatively little of the host IgG present in replete *I. scapularis* nymphs was present in the ticks after molting to the adult stage (Table 2). Similarly, host IgG was absent from the hemolymph and at very low concentrations in the bodies of unfed *I. scapularis* nymphs, suggesting that host IgG obtained from the larval blood meal was also metabolized or excreted.

In contrast, *D. variabilis* maintained host IgG in hemolymph and whole body homogenates (i.e., midgut) throughout nymphal development (Table 1). Presumably, host lgG present in the body and hemolymph of unfed nymphs was acquired from the preceding larval instar. Thus, the presence of host IgG in the hemolymph and bodies of both unfed nymphs and newly molted adults suggests that *D. variabilis* ticks maintain host IgG throughout both larval and the nymphal molts. Studies utilizing a rat and a rabbit as separate hosts for larval and nymphal ticks demonstrated that interchange of the 2 species-specific IgGs occurs early during the feeding process (Table 3). Within 2 days of attaching to a new host, nymphal ticks had completely rid themselves of the old larval lgG and initiated uptake of IgG from the new host. Experiments by Wang and Nuttall (1994, 1995a, 1995b) provide convincing evidence that the clearance of the "old" IgG is probably accomplished by specific uptake and secretion of host IgG in tick saliva via IgG-binding proteins in the hemolymph and salivary glands. Thus, our findings are consistent with the notion that previously acquired IgG in the hemolymph of host seeking nymphal and adult ticks is probably secreted into the new host soon after tick attachment The mechanism(s) responsible for the rapid clearance of larvally-acquired IgG from whole body homogenates (i.e., presumably the tick digestive tract) of recently attached nymphs is unknown but possibilities include defecation, diuresis, or rapid passage through the gut and excretion through the saliva (viz. immunoglobulin-binding proteins as diagrammed in Wang and Nuttall 1999).

The pattern of IgG processing by *D. variabilis* nymphs is similar to the pattern reported for adult females of the argasid tick, *Onzithodoros moubata* Murray (Chinzei and Minoura 1987). These workers reported that even before adult ticks had fed, IgG was present in the hemolymph. The source of this IgG was attributed to the blood meal taken in the preceding nymphal instar. Immediately after feeding, hemolymph concentrations dropped to almost zero, remained low for 5 days, then increased and leveled off to pre-feeding levels (10–15 ng/μl). Hemolymph IgG persisted at these concentrations for 157 days. The initial decrease in hemolymph IgG concentrations soon after feeding may represent the voiding of the "old" IgG — perhaps through the coxal glands, since adult argasid ticks do not remain attached to the host for more than a few hours. Time course experiments whereby radiolabelled IgG was injected into the hemocoel of *O. moubata* 8 d after feeding showed that only 30% of the radiolabelled IgG was recoverable at 24 hr post-injection, indicating a metabolic turnover of radiolabelled IgG within the hemolymph. Yet, the concentration of unlabelled IgG in the hemolymph from blood feeding remained steady. This suggests that, following initial excretion of old IgG from the previous blood meal, there occurs a continual, perhaps even regulated, influx of IgG from the midgut into the hemocoel, thereby maintaining a homeostatic equilibrium of IgG in the hemolymph of adult *O. moubata* ticks.

It is not known why some tick species (e.g., *D. variabilis* and *O. moubata*) may replenish the IgG in their hemolymph, whereas other species (e.g., *I. scapularis*) apparently clear host IgG from their hemolymph. Indeed, the possible function (if any) of host IgG in tick hemolymph remains a matter of conjecture — possibilities include utilization of host IgG as a nutrient reservoir, an osmotic effector or perhaps even an inimune function. Nevertheless, at least 2 practical implications emerge from these studies. First, the relative proclivity of a tick species to either maintain or clear host IgG from its body during the immature stages may affect the efficacy of anti-tick vaccine efforts. Anti-tick vaccines designed to target antigens in the tick hemocoel (see Sauer et al. (1994)) are more likely to succeed against those tick species in which hemolymph IgG persists or is replenished. Second, transstadial persistence of hemolymph IgG may be exploited in ecological studies to determine the host feeding patterns of immature ticks. By assaying the hemolymph of questing nymphs and adults for host-specific IgGs (e.g., bird, lizard, etc.), it may be possible to determine the identity of the previous host blood meal of individual ticks. Furthermore, if this approach is used in conjunction with pathogen testing (e.g., polymerase chain reaction [PCR]; see Higgins and Azad (1995)), it may be possible to determine the host species responsible for infecting individual wild-caught ticks. Unfortunately, the identification of a tick's previous host bloodmeal via residual host IgG is not likely to succeed with tick species that rapidly clear host IgG upon molting (e.g., *I. scapularis*). With such tick species, an alternate strategy may be the use of PCR techniques to identify residual fragments of host cytochrome b DNA (Kirstein and Gray 1996).

This is the first detailed study of IgG uptake and utilization in nymphal ticks. As with many exploratory studies, this study raises several new questions. For example in our kinetic studies, tick species were fed on different hosts (i.e., *D. variabilis* on

rat; *I. scapularis* on rabbit). There are some differences in IgG amino acid sequences between rat and rabbit — particularly in the kappa variable region (see Sitnikova and Nei (1996)). Could such differences account for the very different ways in which host IgG was utilized by *D. variabilis* verus *I. scapularis*? If so, this would be extremely interesting, considering that the nymphs of both these tick species parasitize an even larger variety of host species in nature. Is the general pattern of host IgG utilization by immature ticks determined by tick species, host species or perhaps an interplay of both? In terms of tick-host co-evolution, it may be worthwhile to know the general kinetics of IgG utilization for different tick species fed on very different host species, such as mammals versus reptiles versus birds. Also, it remains unknown whether or not ticks are capable of actively regulating host IgG levels in their hemolymph during inter-feeding periods and, if so, for what purpose? Clearly, more work is needed to understand in detail the basic processes involved in tick metabolism of IgG, including the extent of transstadial persistence of hemolymph IgG among tick species and the physiological function of host IgG in both argasid and ixodid ticks.

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