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A Novel Mode of Arbovirus Transmission Involving a Nonviremic Host

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In nature, infected and uninfected arthropod vectors often feed together on an animal. In mimicking this scenario in the laboratory, uninfected vectors were found to acquire virus while cofeeding on the same host as infected vectors. However, the vertebrate host on which they fed did not develop detectable levels of virus in its blood. These observations were made with Thogoto virus, an influenza-like virus of medical and veterinary significance. *Rhipicephalus appendiculatus* ticks were used as the vector and guinea pigs as the vertebrate host. The results demonstrate that a vertebrate that is apparently refractory to infection by an arthropod-borne virus can still play an important role in the epidemiology of the virus, and they suggest a novel mode of arthropod-borne virus transmission.

THE WORLD HEALTH ORGANIZATION defines arboviruses (arthropod-borne viruses) as "viruses which are maintained in nature . . . through biological transmission between susceptible vertebrate hosts by hematophagous arthropods; they multiply and produce viremia in the vertebrates, multiply in the tissues of arthropods, and are passed on to new vertebrates by the bites of arthropods after a period of extrinsic incubation" (*1*). Biological transmission of an arbovirus is confirmed in the laboratory when the arthropod vector becomes infected by feeding on a viremic vertebrate host infected by inoculation or on a virus-containing artificial blood meal. In nature, however, infected and uninfected vectors frequently feed together on the same host. To investigate virus transmission under these conditions, we conducted studies with Thogoto (THO) virus, which is transmitted biologically by the African brown ear tick *Rhipicephalus appendiculatus* (*2*) and is structurally similar to the influenza viruses (family Orthomyxoviridae) (*3*).

Thogoto virus was originally isolated from ticks collected from cattle in Kenya (*4*) and subsequently has been detected throughout central Africa and in parts of the Middle East and southern Europe. The virus is of medical and veterinary significance (*4–6*). A laboratory colony of *R. appendiculatus*, a three-host tick of the family Ixodidae, was established by feeding the ticks on guinea pigs (female Dunkin Hartley strain, average weight 400 g) and maintaining the interfeeding stages at 28°C and a relative humidity of 85%. Donor adult ticks were infected at the preceding nymphal stage with THO virus by feeding on viremic hamsters (*2*) that had been inoculated with either the prototype IIA (*4*) or Sicilian SiAr 126 (*7*) isolates of the virus. Both isolates had been passaged through mouse brain seven times, plaque-picked three times in Vero cell cultures, and then passaged three times in BHK cell cultures. Blood levels of virus were assayed by either plaque titration in Vero cell cultures (*2*) or intracerebral inoculation of 2-day-old mice (Pathology Oxford strain); minimum detectable titers were 20 plaque-forming units (PFU) per milliliter and 1.0 $\log_{10}$ mouse median lethal dose (LD_{50}) per 0.01 ml of blood, respectively.

Infected adult *R. appendiculatus* (donors) were allowed to cofeed on guinea pigs with uninfected ticks (recipients), by placing them either together in the same retaining chamber or in different chambers, on the same animal. In the first experiments, the recipients were uninfected adults (on guinea pigs GP1 to GP4; Table 1). After engorgement, 76% of the recipient adults that were physically separated from the donor ticks had acquired THO virus (chambers 2 of GP1 and GP2). Of the recipient adults sharing the same chamber as donor adults (chambers 1, GP1 to GP4), of either the same or different sex, 64% acquired the virus. A higher proportion of recipient females (81%) compared with males (55%) became infected. There was no evidence of transvenereal infection either from male to female or vice versa. The experiment was repeated with the use of either uninfected nymphs or larvae as recipients (GP5 and GP6; Table 1): 72% of the nymphs (on GP5) and all of three pools of larvae (on GP6), assayed 12 or 10 days after engorgement, respectively, had become infected. During the period of engorgement, not one of the guinea pigs tested developed a viremia detectable by plaque assay of whole blood diluted in medium. However, by day 14, all of them showed serological conversion (50% neutralizing antibody titers of 1/32 to 1/128). The results indicate that THO virus transmission from infected to uninfected ticks can occur while ticks feed on an apparently nonviremic host.

The efficiency of this novel mode of virus transmission on guinea pigs was compared with transmission on hamsters that develop high viremic titers (Table 2). Nymphs cofeeding with infected adult ticks on guinea pigs (GP7 and GP8) had significantly higher infection rates (98%) than nymphs of the same batch fed under similar conditions on two hamsters (68%; $\chi^2 = 10.5$, $P < 0.01$). Virus was not detected in the blood of the guinea pigs (by either plaque assay or mouse inoculation) during the period of nymphal engorgement. Both hamsters, however, developed viremia, H1 reaching a maximum titer of 7.5 $\log_{10}$ mouse LD_{50} (6.0 $\log_{10}$ PFU) per 0.01 ml of blood on day 6 after tick attachment, and H2, 7.9 $\log_{10}$ mouse LD_{50} (5.9 $\log_{10}$ PFU) per 0.01 ml on day 7. Thus vector-borne virus transmission, involving a host seemingly refractory to the virus, was more efficient than transmission via a sensitive host.

To determine if THO virus isolates could replicate in guinea pigs, we inoculated four animals subcutaneously, two with the SiAr and two with the IIA isolates, at doses of 5000 PFU per animal, and then killed them on day 4 or 5 after inoculation [these conditions gave maximum viremic titers in hamsters (*3*)]. Titration by plaque assay of extracts from brain, lung, heart, liver, spleen, kidney, auxillary lymph node, and subcutaneous tissue showed no detectable virus (<10 PFU per whole organ). No virus was demonstrated in the blood by plaque assay, but, when inoculated into 2-day-old mice, blood samples from animals inoculated with the SiAr isolate (but not the IIA isolate) produced clinical signs; virus isolated from the brains of the affected mice was shown to be THO virus by plaque neutralization assay. Blood from the guinea pigs killed 4 and 5 days after inoculation had titers of <1.0

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and 1.0 $\log_{10}$ LD_{50} per 0.01 ml, respectively. These results demonstrate low-level virus replication of the THO SiAr virus isolate in guinea pigs.

To examine whether this low level of replication was sufficient to infect ticks, we inoculated two guinea pigs with 5000 PFU of SiAr isolate, either 5.5 hours before (GP9) or 48 hours after (GP10) attachment of 40 uninfected nymphs. The time of inoculation of GP10 was considered to provide optimum conditions for virus uptake [by extrapolation from studies with hamsters (*2*)], whereas GP9 avoided possible uptake directly from the virus inoculum. After completion of engorgement, 1 out of 18 nymphs on GP9 and 2 out of 18 on GP10 contained virus; 12 days after engorgement, 1 out of 20 and 3 out of 18 nymphs, respectively, were infected. Viremia was not detected by plaque assay. Although a low level of virus replication in guinea pigs was sufficient, therefore, to infect 5 to 17% of the nymphs, it did not account for the high infection rates of nymphs (72 to 100%) that acquired virus while cofeeding with infected adult ticks (on GP5, GP7, and GP8).

Table 1. Oral infection of *R. appendiculatus* ticks cofeeding on guinea pigs with THO virus–infected ticks. Adult ticks infected with THO virus (*10*) were placed on uninfected guinea pigs (GP1 to GP6) either together with or in different retaining chambers from uninfected ticks. When the ticks had completed engorgement and detached from the host, they were homogenized and then assayed for virus by plaque titration in Vero cell culture (*2, 11*). Daily blood samples were taken by cardiac puncture (under anesthetic) from GP3, GP4, GP5, and GP6 and assayed for virus. The guinea pigs were killed 14 days after ticks were first placed on them, and their serum was screened by plaque reduction neutralization tests in Vero cell cultures (*2*). NI, not infected.

Animal*	Chamber	Unfed ticks		Ticks after feeding			
		Tick†	Virus	Day tested	No. tested	No. infected	Percent infected
GP1	1	16 M	NI	0	8	5	63
		16 F	IIA	0	11	11	100
	2	8 M	NI	0	3	2	67
		8 F	NI	0	8	8	100
GP2	1	16 M	IIA	0	16	14	88
		16 F	NI	0	8	7	88
	2	8 M	NI	0	7	3	43
		8 F	NI	0	3	3	100
GP3	1	17 M	IIA	0	17	15	88
		16 M	NI	0	13	7	54
GP4	1	16 F	IIA	0	15	14	93
		16 F	NI	0	13	8	62
GP5	1	4 F	SiAr	0	4	4	100
		4 M	NI	0	4	4	100
	2	50 N	NI	0	20	10	50
			NI	12	18	13	72
GP6	1	4 F	SiAr	0	4	4	100
		4 M	NI	0	2	2	100
	2	200 L	NI	0	30	0	0
			NI	10	90	90	100

*GP1 and GP2 each represent pooled results from duplicate guinea pigs; all other results are for single animals. †Numbers of adult males (M) and females (F), nymphs (N), and larvae (L).

Table 2. Oral infection of *R. appendiculatus* nymphs cofeeding with THO virus–infected ticks on guinea pigs and hamsters. Adult female ticks infected with THO virus (*10*) were placed together with uninfected male ticks in chambers 1 of either uninfected guinea pigs (GP7 to GP8) or hamsters (H1 and H2). Cohorts of 50 uninfected nymphs, all from the same batch, were each placed on the four animals, in chambers 2. Virus assays were carried out as described (*11*). After feeding, mean virus titers for donor females were 4.1 (range, 2.9 to 4.9) $\log_{10}$ PFU per tick; recipient males were 3.5 (1.0 to 4.5), and recipient nymphs, 3.9 (1.0 to 5.2) $\log_{10}$ PFU per tick. There was no difference in virus titers of recipient ticks fed on either guinea pigs or hamsters.

Animal*	Chamber	Unfed ticks		Ticks after feeding			
		Tick†	Virus	Day tested	No. tested	No. infected	Percent infected
GP7	1	4 F	SiAr	0	3	3	100
		4 M	NI	0	3	3	100
	2	50 N	NI	12	20	19	95
GP8	1	4 F	SiAr	0	3	3	100
		4 M	NI	0	3	2	67
	2	50 N	NI	12	20	20	100
H1	1	4 F	SiAr	0	4	4	100
		4 M	NI	0	3	3	100
	2	50 N	NI	12	20	15	75
H2	1	4 F	SiAr	0	3	2	67
		4 M	NI	0	3	3	100
	2	50 N	NI	12	20	12	60

*GP7 and GP8 each represent pooled results from duplicate guinea pigs; all other results are for single animals. †Numbers of adult males (M) and females (F) and nymphs (N).

Arboviruses represent the largest ecological group of viruses [some 500 have been described (*8*)]. By definition (*1*), the prerequisite for acquisition of an arbovirus by its arthropod vector is that the host on which the vector feeds is viremic. In this study we showed that tick-borne transmission of THO virus can occur without the requirement for a viremic host. All three stages of *R. appendiculatus* acquired virus while cofeeding with infected ticks on an apparently nonviremic host. The efficiency of virus transmission in this manner was greater than that involving a viremic host.

The significantly higher infection rates of cofeeding ticks, compared with ticks fed on inoculated guinea pigs, suggest a novel mode of virus transmission independent of viremia. Natural clustering of ticks on a host (*9*) during feeding may facilitate this mode of transmission. Several experiments were carried out to investigate the mechanism of virus transmission involving guinea pigs. Recipient nymphs were shown to require at least 4 days of cofeeding with infected adults in order to become infected; the distance between infected and uninfected ticks feeding together on the same guinea pig did not affect the number of recipient ticks that acquired virus. These results indicate that virus transmission was not due to passive drainage of virus from the site of feeding of infected ticks but that virus replication in the guinea pig was a prerequisite of transmission.

The observation that arbovirus transmission can occur at high frequency when infected and uninfected ticks cofeed on a nonviremic host has important implications. It demonstrates that a host permissive for the vector, and apparently nonpermissive for the virus, can play a significant role in the epidemiology of an arbovirus; such animals would be considered unimportant when evaluated by currently accepted criteria, that is, development of a detectable viremia. The results also suggest a possible mechanism of virus transmission that does not rely on development of a viremia. The significance of these findings with regard to the trans-

mission of other arboviruses, particularly those transmitted by long-feeding ticks, needs to be explored.

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10. Donor ticks were infected at the nymphal stage by feeding on viremic hamsters inoculated with either the prototype IIA or Sicilian SiAr 126 THO virus isolates and then reared to the adult stage; average titers for males and females were 2.0 and 3.0 for IIA and 2.5 and 3.0 for SiAr, $\log_{10}$ PFU per tick, respectively.
11. Adults were assayed individually for virus (Table 1) on the day they completed engorgement (0), nymphs individually on days 0 and 12, and larvae in three pools of 10 on day 0 and in three pools of 30 on day 10 [days 12 and 10 were the times of maximum virus titers, respectively (*2*)]. Mean virus titers for donor females after feeding were 4.1 (range, 2.0 to 5.4), and 2.6 (1.7 to 3.7) $\log_{10}$ PFU per tick for donor males; recipient females were 3.9 (1.1 to 5.4), recipient males, 2.2 (1.3 to 3.7), recipient nymphs, 2.2 (1.3 to 3.0) on day 0 and 4.3 (3.4 to 4.9) on day 12, and recipient larvae <1.0 on day 0 and 1.4 (0.9 to 1.8) $\log_{10}$ PFU per tick on day 10 after engorgement. There was no difference in virus titers of recipient ticks that became infected with either IIA or SiAr THO virus.
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Heparin Promotes the Inactivation of Antithrombin by Neutrophil Elastase

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Heparin is an acceleratory cofactor for antithrombin, a circulating inhibitor of blood coagulation enzymes. The presence of heparin on blood vessel walls is believed to contribute to the nonthrombogenic properties of those surfaces. In apparent opposition to this function, heparin was found to greatly accelerate the in vitro inactivation of antithrombin by neutrophil elastase. Inactivation rates in solution were potentiated several hundredfold by specific heparin fractions with anticoagulant activity. Although the data suggest that a heparin-antithrombin complex is essential for the inactivation by elastase to occur, the enzyme itself interacts tightly with heparin. These results suggest a mechanism which, if operating in vivo, could lead to a localized neutralization of the anticoagulant function of heparin at the endothelial surface.

THE BLOOD COAGULATION SYSTEM IS a series of linked proteolytic reactions that, in the presence of appropriate stimuli, can rapidly lead to clot formation. A number of natural anticoagulant mechanisms are present to guard against the undesirable deposition of thrombi within normal blood vessels. Recent evidence suggests that the luminal surface of the blood vessel, the endothelium, is itself actively involved in the regulation of thrombosis (*1*). When the normally nonthrombogenic nature of the endothelium is lost, as in certain inflammatory conditions, a hypercoagulable state can result. The current study suggests a mechanism by which the normal functioning of the endothelial heparin-antithrombin system might be modified in inflammatory disease.

Antithrombin (also referred to as antithrombin III or AT-III) is the primary plasma inhibitor of blood coagulation enzymes and is a member of a large superfamily of related proteins that includes several serine proteinase inhibitors (serpins) (*2*). These proteinase inhibitors share a common mechanism by which a covalent, essentially irreversible complex is formed with the target enzymes (*3*). It is interesting that several of these inhibitors are themselves the target of attack by proteinases of mammalian and nonmammalian origin. In the case of antithrombin, neutrophil elastase catalyzes a specific and inactivating cleavage near the site involved in the inhibition of coagulation enzymes (*4*). It has been hypothesized that this inactivation phenomenon contributes to the hypercoagulable state linked to neutrophil activation in inflammation and septicemia (*5, 6*).

Maintenance of plasma concentrations of antithrombin at or near the normal level of approximately 2 μM is apparently essential to avoid the thrombotic tendency associated with both hereditary and acquired deficiency states (*7*). Disease-related decreases in circulating antithrombin levels can be more than 50% in extreme cases of septicemia. Such large decreases have most often been attributed to a massive consumption of antithrombin in the course of inhibiting large amounts of activated coagulation enzymes. A possible alternative explanation was suggested by the recently described inactivation of antithrombin in vitro by neutrophil elastase (*5*). The widespread activation of neutrophils in inflammation and the potential for significant release of elastase give this mechanism some appeal. However, the extrapolation of this observation to the physiological setting must take into account the very high plasma levels of a specific and fast-acting elastase inhibitor. This inhibitor, α-1 proteinase inhibitor, normally prevents expression of elastolytic activity in plasma (*3*). To address this conceptually problematic situation, we investigated the influence of heparin on the elastase-antithrombin interaction.

The anticoagulant heparin is a functionally and chemically heterogeneous sulfated carbohydrate with the ability to bind to antithrombin and accelerate its rate of inhi-

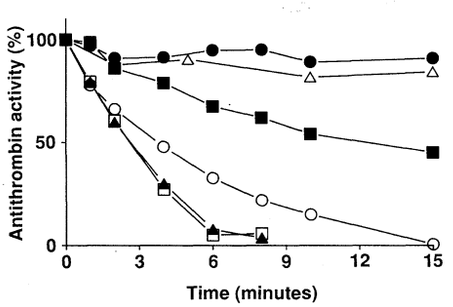

Fig. 1. Effect of heparin on the rate of antithrombin inactivation by neutrophil elastase. A reaction mixture that contained purified human antithrombin (*10*) at a concentration of 2 μM and various concentrations of heparin in 0.15M NaCl and 0.02M tris HCl, pH 7.5, was warmed to 37°C. Elastase was added to a concentration of 5 nM to initiate the reaction. Reactions were stopped at the indicated times by a 20-fold dilution of an aliquot of the reaction mixture into the second-phase assay buffer (0.15M NaCl and 0.05M tris HCl, pH 8.4) that contained 1 μg of α-1 proteinase inhibitor per milliliter. Antithrombin activity was then determined by a chromogenic heparin cofactor assay as described (*7*). The individual reaction mixtures were as follows: no added heparin (●); 1 μg per milliliter of inactive heparin (△); 50 (■), 100 (○), 400 (▲), or 700 (□) nanograms per milliliter of the anticoagulant fraction of heparin. The human neutrophil elastase was a gift of J. Travis or was a human sputum–derived preparation obtained commercially from Elastin Products Company.

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