

pH-optimum urease must be shielded from gastric acidity and prevented from being active at neutral pH to avoid lethal alkalization (5). Urea transport via UreI allows the internal urease of *H. pylori* to generate ammonia in an acid environment, buffering the periplasm. This allows the organism to survive and grow in the stomach in the presence of usual gastric urea concentrations. The absence of transport by UreI at neutral pH prevents high urease activity in the absence of gastric acidity, as occurs during digestion. The combination of a high level of a neutral pH-optimum urease and an acid-regulated urea channel explains why *H. pylori* is unique in its ability to inhabit the human stomach. Effective inhibition of UreI would provide a means of eradicating the organism in the normal, acid-secreting stomach.

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12. A poly(A) cassette (a region of repeated adenosine) was cloned into pcDNA3.1⁺ (Invitrogen, Carlsbad, CA). The *ureI* gene sequence was inserted upstream of the poly(A) cassette and downstream of the pcDNA3.1 T7 promoter. cRNA was prepared using the mMessage mMachine in vitro transcription system (Ambion, Austin, TX). Fifty nanoliters of cRNA (1 µg/µl) was injected and oocytes were maintained at 18°C in Barth's solution for 3 days before use in uptake experiments.
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19. In vitro transcription/translation was done with TNT rabbit reticulocyte lysate in the presence of ³⁵S-methionine, with or without canine pancreatic microsomes (Promega, Madison, WI). A fusion vector, UreIN-ter, was engineered in pcDNA3.1⁺, based on a system previously used to analyze the topology of several integral membrane proteins [D. Bayle, D. Weeks, G. Sachs, *J. Biol. Chem.* **272**, 19697 (1997)]. Progressively longer sections of UreI, each starting with the NH₂-terminus, were fused to a COOH-terminal glycosylation flag taken from the COOH-terminal 177 amino acids of the H⁺, K⁺-ATPase β subunit, containing five N-linked glycosylation consensus sequences. Glycosylation indicates translocation of the COOH-terminus into the microsomal membrane.
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22. *H. pylori* were grown overnight from glycerol stock on blood agar plates (Baxter, Irvine, CA). *Helicobacter pylori* were disrupted by successive passages through a French pressure cell at 10,000 psi. Urease activity, detected as evolved ¹⁴C-CO₂, was measured as previously described (5).
23. Antibody generation and affinity purification were carried out by Alpha Diagnostics International (San Antonio, TX). The epitopes UP1 (CEGAEDIAQVSHHLTNFYGPATG) and UP2 (CAILSHYSMDLDDHKVLGITEGD) (24), are within the first and second periplasmic loops. Homogenate and membranes were resolved on 10% SDS-tricine gels. Proteins were transferred to either nitrocellulose (Bio-Rad, Hercules, CA) or polyvinylidene difluoride (Millipore, Bedford, MA). After transfer, blots were blocked by incubation in a 5% solution of nonfat dry milk in phosphate-buffered saline-Tween for 1 hour. The membranes were incubated with antibodies to UP2 at a 1:2000 dilution in blocking solution. Binding was detected using a peroxidase-coupled rabbit antibody to immunoglobulin G at 1:20,000 dilution (American Qualex, San Clemente, CA) with ECL or ECL plus (Amersham, Arlington, IL).
24. Reaction buffers contained 100 mM NaCl, 2 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, and 10 mM MES for pH values ≤6.0 or 10 mM Hepes for pH values ≥6.0. Before each experiment, the oocytes were transferred from Barth's solution to a reaction buffer (pH 7.0) at 21°C. A reaction was started with the transfer of 5 to 7 oocytes to the reaction buffer at 21°C that contained a labeled compound, such as ¹⁴C-urea, and was terminated with the transfer of the oocytes to an ice-cold buffer (pH 7.5). Each oocyte was individually dissolved with SDS and mixed with scintillation cocktail for counting of the labeled compounds.
25. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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Transmission of Vesicular Stomatitis Virus from Infected to Noninfected Black Flies Co-Feeding on Nonviremic Deer Mice

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Vesicular stomatitis is an economically important arboviral disease of livestock. Viremia is absent in infected mammalian hosts, and the mechanism by which insects become infected with the causative agents, vesicular stomatitis viruses, remains unknown. Because infected and noninfected insects potentially feed on the same host in nature, infected and noninfected black flies were allowed to feed on the same host. Viremia was not detected in the host after infection by a black fly bite, but because noninfected black flies acquired the virus while co-feeding on the same host with infected black flies, it is concluded that a viremic host is not necessary for an insect to be infected with the virus. Thus co-feeding is a mechanism of infection for an insect-transmitted virus.

Vesicular stomatitis is an arthropod-borne viral disease that primarily affects cattle, swine, and horses; it causes vesicular lesions on the mouth, coronary bands, and teats. Many species of wildlife and humans are also at risk. The causative agents, vesicular stomatitis viruses (VSVs), are a group of antigenically related but distinct viruses of the genus *Vesiculovirus*, family Rhabdoviridae (1).

Despite intensive study, aspects of the epizootiology of VSVs, including modes of transmission and endemic maintenance, remain largely unknown and highly controversial. The World Health Organization (WHO) definition of an arbovirus (2) implies that only vertebrate species that develop detectable viremia after infection are significant in the epidemiology of these viruses and stipulates that vector infection

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is through ingestion of the blood of an infected vertebrate host. This assertion is problematic for VSVs because the virus reportedly does not produce viremia in mammalian hosts (3). To date, viremia has not been documented for any wild or domestic animal species naturally or experimentally infected with VSVs (4), and the mechanism by which insects become infected in nature remains unknown.

Serologic studies conducted within epizootic and enzootic regions of the United States and throughout the Americas have demonstrated that rodents are naturally infected with VSV New Jersey serotype (VSV-NJ). Antibodies to VSV-NJ have been detected in rodents in Colorado (5), Louisiana (6), Costa Rica (7), and Mexico (8). Because sand flies (*Lutzomyia* spp.) are known vectors of VSV-NJ and are often found to be nest associates of rodents (9), a VSV-NJ enzootic cycle with rodents serving as the virus reservoir in nature is hypothesized.

Previous experimental infections of rodents with VSV-NJ involved parenteral inoculation of VSV-NJ into laboratory rodents (10) and may not result in the spectrum of clinical signs associated with infection by insect bites in wild rodents (11). During blood feeding of hematophagous arthropods, a variety of molecules are secreted in the saliva that counteract the host hemostatic response to disruption of the dermal vascular system. Modulation of the host immune system by vector saliva provides an opportunity for enhancing transmission of the pathogen during blood feeding and may influence the course of infection (12, 13).

In this study, we allowed VSV-NJ-infected black flies (*Simulium vittatum*) to feed on susceptible deer mice to confirm their vector competence and to determine whether viremia is present in deer mice after they are exposed to VSV-NJ. Because noninfected and infected vectors often feed together on the same host in nature, we allowed noninfected "recipient" black flies (14) to co-feed with infected black flies to determine whether virus was transferred from one fly to the other while they were feeding on the same host. Recipients were not caged together but were kept in individual snap cap tubes. A relatively high percentage of recipient flies were positive, indicating acquisition of the virus by co-feeding transmission. Although transmission during co-feeding has been reported for several species of ticks and for tick-borne viruses (15), this phenomenon has not been reported previously for an insect-transmitted virus.

We chose *S. vittatum* as the donor in this study because it feeds on a variety of hosts and

Table 1. Infection of recipient flies by co-feeding on 6-week-old *P. maniculatus* with VSV-NJ-infected black flies

| Mouse | No. of infected flies feeding | No. of recipient flies (no. positive) |
|-------|-------------------------------|---------------------------------------|
| 1-1 | 2 | 1 (0) |
| 1-2 | 2 | 2 (1) |
| 1-3 | 1 | 2 (0) |
| 1-4 | 3 | 1 (0) |
| 1-5 | 1 | 1 (0) |
| 1-6 | 4 | 1 (0) |
| 1-7 | 2 | 0 (0) |
| 1-8 | 1 | 2 (0) |
| 1-9 | 2 | 1 (1) |
| 1-10 | 2 | 2 (0) |
| 1-11 | 3 | 3 (1) |
| 1-12 | 1 | 2 (0) |

is a major pest of livestock (16, 17). VSV-NJ was isolated from *S. vittatum* during the 1982–1983 U.S. epizootic (18), and this species was later shown to be a competent laboratory vector of VSV-NJ (19). We chose to use the deer mouse (*Peromyscus maniculatus*) because it has been shown to be naturally infected with VSV-NJ in epizootic regions (5) and therefore is a potential amplifying host during epizootics.

We infected donor black fly females with VSV-NJ by intrathoracic inoculation (20). Time 0 infection titers in donor black flies ranged from 2.2×10^2 to 3.8×10^3 plaque-forming units (pfu) per fly (mean 2.8×10^3). After a 7-day incubation period, five of six donor saliva samples tested were positive for virus. Saliva virus titers ranged from 2.5×10^1 to 4.8×10^3 pfu/ml and whole body titers were all $>1 \times 10^4$ pfu per fly.

We used 6-week-old and 6-month-old female deer mice (wild type; *Peromyscus* Genetic Stock Center, Columbia, South Carolina) in two independent trials (21). For each trial, mice were randomly allocated to one of two groups: group 1 consisted of mice that were fed upon by donor black flies and group 2 consisted of mice that were injected intramuscularly (im) with 0.2 ml of a VSV-NJ suspension (4.0×10^6 pfu/ml). Mice were housed in microisolation cages (four mice per cage) at 22°C.

Fourteen 6-week-old female mice were used in trial 1. Twelve mice were included in group 1, and two mice were in group 2. We allowed as many as four donor black flies to feed from each group 1 mouse (Table 1). Donor feeding times varied from 2 to >30 min.

We did not detect any virus in the blood of any of the group 1 or group 2 mice between days 1 and 4 postinfection (PI). Eighteen recipients fed to repletion from group 1 mice during trial 1 and were assayed for virus by tissue culture inoculation. We recovered virus from three recipients. The amount of virus ingested by each was not determined; however, we detected virus titers between 3.8×10^3 and $1.7 \times$



Fig. 1. Donor (bottom) and recipient (top) black flies co-feeding on *P. maniculatus*. Recipient black flies were marked on the thorax with a drop of white correction fluid.

10^4 pfu per fly after 6 days of incubation. No recipients fed from group 2 mice.

Ten 6-month-old female deer mice were used in trial 2. Nine mice were in group 1, and one mouse was in group 2. Between one and three donors fed on each group 1 mouse (Table 2). Fourteen recipients co-fed during the initial infection period, 15 fed at 24 hours, and 12 fed at 48 hours PI. Seven recipients fed from the group 2 mouse (Table 2, mouse 2-10) between 24 and 48 hours PI.

Viremia was not detected in blood samples collected from individual mice between days 1 and 4 PI. Virus was recovered from 14%, 26%, and 25% of the recipients feeding on group 1 mice at time 0, at 24 hours, and at 48 hours PI, respectively, during trial 2. Virus was not recovered from the recipient black flies feeding on the group 2 mouse in this trial.

Even though VSV-NJ has been isolated from arthropods (including black flies) collected in enzootic areas and during epizootics, doubt has remained about their role in natural transmission cycles because all the potential vertebrate hosts investigated to date have failed to develop a viremia sufficient to infect biting arthropods. Previous investigations of potential reservoir hosts have involved parenteral inoculations of laboratory rodents and wild animals (4, 10). When a viremia was not detected in the animal in question, it was considered to be a nonfactor in the maintenance of VSV-NJ.

Peromyscus maniculatus does not meet the standard definition of a reservoir host for VSV-NJ. Regardless of route of exposure (black fly bite or im injection), we did not

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Table 2. Infection of recipient black flies co-feeding on 6-month-old *P. maniculatus* with VSV-NJ-infected black flies.

| Mouse | Time of feeding PI | | | | | |
|-------|-------------------------------|---------------------------------------|-------------------------------|---------------------------------------|-------------------------------|---------------------------------------|
| | Time 0 | | 24 hours | | 48 hours | |
| | No. of infected flies feeding | No. of recipient flies (no. positive) | No. of infected flies feeding | No. of recipient flies (no. positive) | No. of infected flies feeding | No. of recipient flies (no. positive) |
| 2-1 | 2 | 1 (0) | 2 | 1 (0) | 1 | 1 (0) |
| 2-2 | 2 | 2 (0) | 1 | 2 (1) | 1 | 1 (0) |
| 2-3 | 2 | 2 (1) | 3 | 2 (2) | 2 | 2 (1) |
| 2-4 | 2 | 1 (0) | 1 | 2 (1) | 1 | 1 (1) |
| 2-5 | 1 | 0 (0) | 2 | 1 (0) | 1 | 1 (1) |
| 2-6 | 3 | 2 (1) | 2 | 2 (0) | 1 | 2 (0) |
| 2-7 | 3 | 2 (0) | 1 | 1 (0) | 2 | 1 (0) |
| 2-8 | 2 | 3 (0) | 1 | 2 (0) | 1 | 1 (0) |
| 2-9 | 1 | 1 (0) | 2 | 2 (0) | 1 | 2 (0) |
| 2-10* | — | — | — | 3 (0) | — | 4 (0) |

*Injected with 0.2 ml of VSV-NJ.

detect viremia by conventional tissue culture methods. However, because recipient black flies became infected while co-feeding with infected black flies on the same nonviremic host, this rodent species should be considered an important maintenance host for the virus in VSV-NJ epizootic and enzootic regions.

Our inability to isolate VSV-NJ from blood is consistent with findings from other investigations (4, 10) and demonstrates the limitations of the WHO criteria, which suggest that all potential vertebrate reservoirs of a virus can be identified by simply screening for viremic hosts. Our results suggest that nonviremic hosts may play an important role in the maintenance of VSV-NJ and that many more vertebrates could serve as maintenance hosts for this virus in nature.

Efficient transmission of VSV-NJ was demonstrated between infected and noninfected black flies co-feeding on the same nonviremic host. Overall, of the 32 recipient black flies co-feeding at time 0, 15.6% became infected. In trial 2, 14.2% of the recipient black flies co-feeding at time 0 became infected and the proportion of positive recipient black flies increased over time. Seven of 27 (26%) black flies became infected when co-feeding with infected black flies between 24 and 48 hours after initial exposure. The increase in the proportion of infected recipients over time may indicate that a salivary component enhances virus transmission, as first suggested by Cupp and Cupp (12).

Field studies have shown that small and large mammals have serum antibodies to VSV-NJ, indicating their presence in the normal chain of infection. The association between black flies and mammals, including livestock and rodents, is well documented (17, 22). Black flies could act as a transfer vector between nonviremic vertebrate reservoirs of VSV-NJ and domestic livestock. Because black flies ex-

hibit site feeding preferences and are often found feeding in close association in large numbers from specific anatomical sites on livestock (16, 23), we propose that nonviremic transmission of VSV-NJ from infected to noninfected black flies co-feeding on the same livestock host may occur.

These results demonstrate that a western U.S. insect species can become infected with VSV-NJ by feeding on a host, and, in fact, that a co-feeding infection mechanism exists for an insect-transmitted virus. Collectively, these results have major significance with regard to the maintenance and transmission of VSV-NJ in enzootic and epizootic regions and help explain how VSV-NJ survives in nature when susceptible hosts produce minimal or no viremia.

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20. The VSV-NJ isolate used was one we isolated from *Culicoides* sp. collected along the Rio Grande River near Belen, New Mexico, in 1997. The virus was plaque purified and passed twice in Vero-M cells. We used 1- to 2-day-old *S. vittatum* females (IS-7 cytotyping) from the F₁₃₂ and F₁₃₃ generations of a continuous laboratory colony [M. J. Bernardo, E. W. Cupp, A. E. Kiszewski, *Ann. Entomol. Soc. Am.* **79**, 610 (1986)] for these experiments. Because *S. vittatum* is a competent laboratory vector (79), female black flies were infected by intrathoracic inoculation with 1 µl of a VSV-NJ suspension containing 5.0 × 10⁶ pfu/ml. We determined infection rates and viral counts in six black flies immediately after injection by a previously described plaque method [C. J. Maré and D. L. Graham, *Infect. Immun.* **8**, 118 (1973)]. We held the remaining infected black flies (donors) in individual 5-ml snap cap tubes for 7 days at 30°C to allow for virus replication and dissemination. To confirm that virus was in the saliva of donors, we collected saliva by allowing six individually tethered donors to feed into capillary pipettes containing 10 µl of Iscove's modified Dulbecco's medium (Omega Scientific, Inc.) with 20% newborn calf serum and 2% antibiotics (Omega Scientific, Inc.) [M. J. Muller, *J. Med. Entomol.* **24**, 206 (1987)]. We then evaluated individual donors and saliva samples for the presence of virus by a plaque assay. We allowed donor black flies (up to two at a time) to feed from each mouse in group 1. When a donor fed to repletion or showed no interest in feeding, it was removed from the mouse and replaced with a new donor.
21. The use of *P. maniculatus* was approved by the University of Arizona's Animal Care and Use Committee (control 97-096). We collected blood samples (25 µl) from each mouse by tail clip for the first 4 days PI to detect viremia by virus reisoletion in tissue culture.
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