The Aphid Salivary Gland Basal Lamina as a Selective Barrier Associated with Vector-Specific Transmission of Barley Yellow Dwarf Luteoviruses

F. E. Gildow and S. M. Gray

First author, Department of Plant Pathology, Pennsylvania State University, University Park 16802, and second author, USDA/ARS, Department of Plant Pathology, Cornell University, Ithaca, NY 14853.
This research was funded by USDA Competitive Research Grant 90-37151-5275.
Contribution 1942, Department of Plant Pathology, Pennsylvania State University.
Accepted for publication 21 July 1993.

ABSTRACT

Gildow, F. E., and Gray, S. M. 1993. The aphid salivary gland basal lamina as a selective barrier associated with vector-specific transmission of barley yellow dwarf luteoviruses. Phytopathology 83:1293-1302.

The inability of virions of the MAV barley yellow dwarf luteovirus to penetrate the extracellular basal lamina surrounding the accessory salivary glands of some nonvector aphid species suggests that the basal lamina may possess a selective function that regulates vector-specific luteovirus transmission. When *Sitobion avenae* and *Rhopalosiphum padi* were fed for 2 wk on either MAV- or RPV-infected oats and then examined by electron microscopy, virions of both the transmitted MAV and the nontransmitted RPV were observed penetrating the salivary basal lamina of *S. avenae*. When *R. padi* were examined, virions of transmitted RPV consistently were observed in the salivary basal lamina; however, virions of nontransmitted MAV were not observed in the salivary basal lamina of 50 aphids examined from five experiments. When 0.5–2 ng of purified MAV was injected into individual aphids and the aphids were examined by transmission electron microscopy after a 24-h feeding on oats, virions were observed in salivary basal lamina of *S. avenae* and of *Metopolophium dirhodum*, but MAV virions were rarely observed in *R. padi, R. maidis,* or in *Schizaphis graminum*. Virions per unit basal lamina length were counted to determine relative affinity of MAV for salivary basal lamina. The average numbers of MAV virions in basal lamina of *S. avenae, M. dirhodum, R. padi, R. maidis,* and *S. graminum* were 47, 28, <1, 0, and <1 virions per 10 μm of basal lamina length, respectively. When purified MAV virions were treated with any of three MAV-specific monoclonal antibodies or their Fab fragments and injected into *S. avenae,* virions were unable to penetrate the salivary basal lamina. In addition, virions of brome mosaic virus did not penetrate the salivary basal lamina of *R. padi* when each aphid was injected with 30 ng of BMV. These results are consistent with the hypothesis that the accessory salivary gland basal lamina functions as a transmission barrier and that specific virus capsid-glycoprotein interactions regulate basal lamina penetration.

Additional keywords: basement membrane, endocytosis.

The barley yellow dwarf luteoviruses (BYDV) are serologically distinct viruses that cause similar symptoms of leaf yellowing and stunting in small grains and grasses worldwide (20,21). The different BYDVs were initially identified by the fact that they could be transmitted only by specific species of aphids (18). More recent studies have shown that the BYDVs can be divided into two major groups on the bases of serological similarities and genomic structure. The group 1 BYDVs, identified as MAV, PAV, and SGV, are serologically closely related to each other but are serologically distinct from the group 2 BYDVs, RPV and RMV (19). In addition, BYDVs in group 1 differ significantly in nucleotide sequence and genomic organization from those in group 2 (14).

The BYDVs are transmitted in a circulative-nonpropagative

This article is in the public domain and not copyrightable. It may be freely reprinted with customary crediting of the source. The American Phytopathological Society, 1993.

manner by aphids (9,16). One of the most interesting characteristics of the BYDVs is their high level of vector specificity. Although over 20 species of aphids have been reported as vectors (12), each BYDV is efficiently transmitted only by one or a few specific species. The route of luteoviruses through the aphid vector has been identified (3). To be acquired, the virus must be ingested by a feeding aphid from infected phloem, transported through the hindgut epithelium, and released into the hemocoel (4). Although luteovirus recognition regulating virus uptake probably occurs at the hindgut, little selectivity among luteoviruses occurs at this site. Once BYDV enters the aphid hemocoel, it must then pass through the basal lamina and cell membrane (basal plasmalemma) surrounding the accessory salivary gland to be transmitted (6). How virions move through the basal lamina and enter the salivary gland has not been clearly demonstrated. After penetrating the salivary gland, the virus is transported in coated vesicles through the cytoplasm and released into the salivary canal. Once the virus is released into the canal, it can flow with salivary secretions through the salivary duct into host plants during feeding (15).

The high level of luteovirus-vector specificity is believed to be determined by virus recognition at the salivary gland basal plasmalemma. This hypothesis is supported by the discovery that the serologically related BYDVs, MAV and PAV, apparently compete for receptor recognition and subsequent transport through the accessory salivary glands of their common vector *Sitobion avenae* (Fabricius) (5). Furthermore, virions of nontransmitted RPV pass through the salivary basal lamina but do not penetrate the basal plasmalemma of *S. avenae* (6).

In a preliminary study, Gray (8) reported the ability of specific monoclonal antibodies to inhibit transmission of homologous BYDVs when the antibodies were acquired by feeding through membranes or by injection into aphid vectors. Differential effects of antibodies made to specific capsid protein epitopes suggested that different sites of transmission neutralization might be involved. Mechanisms within the aphid associated with neutralization after feeding or after injection were not identified.

Recent studies (*unpublished*) suggested that the extracellular basal lamina surrounding the accessory salivary gland also might play a role in regulating BYDV transmission in some aphid-virus combinations. When *Rhopalosiphum padi* (L.) were fed MAV, which *R. padi* rarely transmits, the virus could not be detected in the salivary basal lamina. This observation was similar to one previously reported by Harris et al (10) in which a nontransmissible isolate of pea enation mosaic virus was not detectable in the salivary glands of injected pea aphids. These observations suggested that the role of the salivary basal lamina in BYDV transmission be reexamined.

The primary objective of our study was to identify sites on the aphid accessory salivary gland that function as virus recognition sites or selective barriers to BYDV transport into the gland and that are associated with transmission specificity. Previous work (5) suggested that receptor-mediated endocytosis at the salivary gland basal plasmalemma was one possible determinate of vector specificity. However, available ultrastructural evidence (3,6) was not convincing. Recent observations (*unpublished*) also suggested a possible role for the extracellular basal lamina that surrounds the accessory salivary gland in regulating luteovirus movement into the gland. Therefore, ultrastructural studies were directed at the examination of luteovirus-salivary gland interactions at the cell basal plasmalemma and basal lamina. A second objective was to determine the mechanism of BYDV transmission neutralization by antibodies injected into the aphid hemocoel. Detailed knowledge of these mechanisms is considered necessary to understand the regulation of virus transmission and could be useful in the development of BYDV control strategies aimed at interfering with the ability of aphid vectors to acquire and transmit BYDV.

MATERIALS AND METHODS

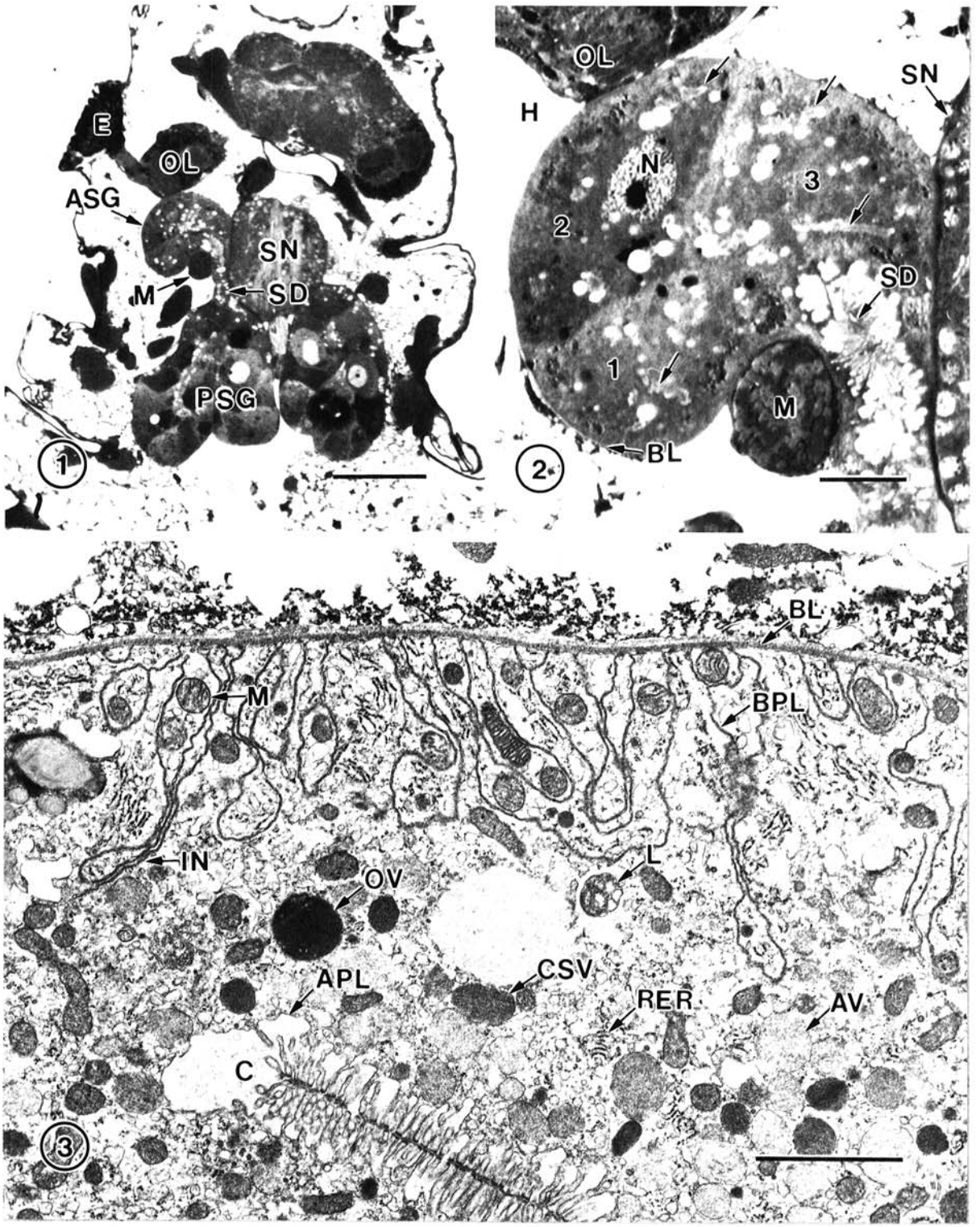
Aphid vector and virus maintenance. Virus-free colonies of the Cornell University clones (18) of *R. padi*, *R. maidis* (Fitch), *Schizaphis graminum* (Rondani), *S.* (formerly *Macrosiphum*) *avenae*, and a California clone (4) of *Metopolophium dirhodum* (Walker) were maintained on caged barley (*Hordeum vulgare* (L.) cv. Black Hulless) in controlled-environment rooms at 20 C with a 24-h photoperiod under fluorescent lighting. Colonies of *M. dirhodum* derived from the original California clone also were reared under similar conditions at Pennsylvania State University. The New York MAV and RPV isolates of BYDV (18) were used for all transmission experiments. MAV is transmitted most efficiently by *S. avenae,* and RPV is transmitted most efficiently by *R. padi.* Oat plants (*Avena byzantina* K. Koch cv. Coastblack) to be used as virus source plants were infested with viruliferous *S. avenae* 1 wk after planting. Following a 48-h acquisition access period on detached leaves from MAV- or RPV-infected oats, aphids were allowed a 5-day inoculation access period (IAP) on seedlings. The seedlings were then fumigated with DDVP (*O,O*-dimethyl-2,2-dichlorovinyl phosphate) to kill the aphids and maintained in a greenhouse for 4 wk prior to use as virus source plants. Enzyme-linked immunosorbent assay to test virus source plants and transmission test seedlings and virion purification for injection were as previously described (3,9).

Virus transmission by feeding and microinjection. Aphid species were compared for the ability to acquire and transmit virus from infected BYDV source plants. Virus-free *S. avenae* and *R. padi* were reared together for 2 wk on the same oat plants infected with either MAV or RPV or on healthy plants as controls. Equal numbers of fourth instar nymphs and apterous adults were then removed, separated by species, and allowed a 24-h IAP on healthy oat seedlings. After the IAP, aphids were removed and prepared for electron microscopic (EM) examination. Test plants were fumigated, placed in a greenhouse, and observed for BYD symptoms for 6 wk.

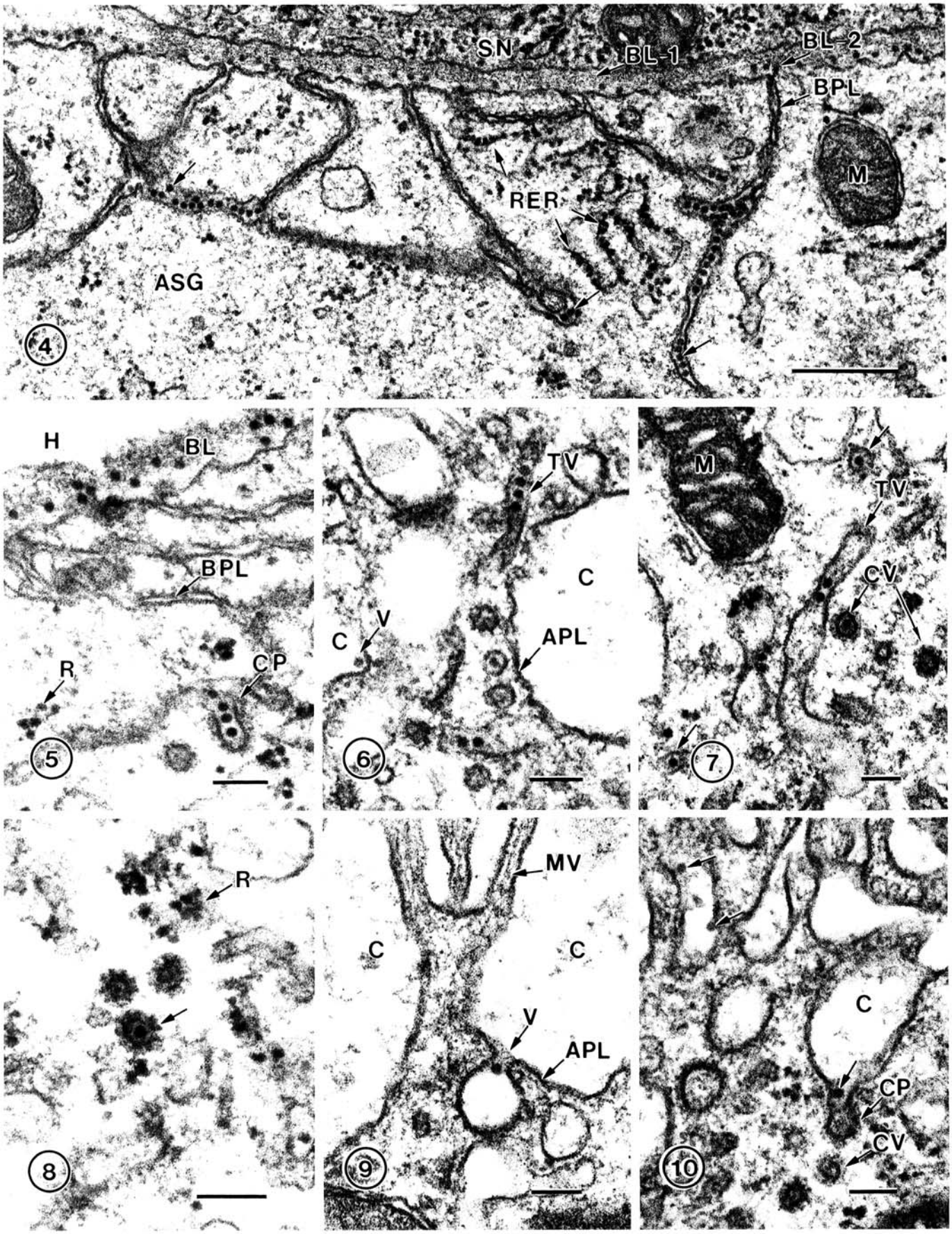
To equalize the concentration of virions among individual aphids and among different species and to enhance visualization of virions at the salivary gland, purified virions were microinjected directly into the aphid hemocoel. Carbon dioxide-anesthetized aphids were injected with approximately 10 nl of purified MAV diluted to 25–100 μg/ml in 0.01 M phosphate buffer (pH 7). Injection volumes were standardized with a Pico-injector apparatus (Medical Systems Inc., Melville, NY) that controlled pressure and time of injection. Individual aphids were then allowed a 24-h IAP on oat seedlings to test for virus transmission prior to being fixed for EM. The seedlings were fumigated, placed in a greenhouse, and observed for BYD symptoms for 6 wk. Selected plants were then tested by enzyme-linked immunosorbent assay (9) to verify virus transmission and isolate identification.

Antibody neutralization studies. Microinjection and EM were used to study the effects of antibody attachment to MAV on the ability of MAV to penetrate the salivary gland basal lamina and plasmalemma and to be transmitted to plants during feeding. Aphids were injected with mixtures consisting of purified MAV virions and anti-MAV monoclonal or polyclonal antibodies (11). The purified IgG fraction of the antibody or IgG Fab fragments (Immunopure kit, Pierce Chemical Co., Rockford, IL) were used in all neutralization experiments. Mixtures consisting of 2.5 μg of virions and 12.5 μg of whole IgG or Fab IgG in 100 μl of sterile water were incubated 1 h at 20 C prior to use. Injection volumes were approximately 10 nl. The ratio of antibody molecules to virus particles was about 200:1, assuming the particle masses of antibodies and MAV were 1.5×10^5 Da and 6.2×10^6 Da, respectively. After injection, aphids were allowed a 24-h IAP on oat seedlings to test for transmission. Individual aphids were then prepared for EM observations of the salivary glands. The plants were fumigated and observed over 6 wk for BYD symptoms.

The ability of IgG or IgG Fab to aggregate virions or destabilize virions was examined for each antibody. Purified MAV virions were mixed with each antibody in the same ratio used in the neutralization studies and incubated for 1 h at 20 C or for 1, 2, or 7 days at 4 C. After each incubation time, the MAV-antibody suspension was centrifuged at 8,000 *g* for 5 min to precipitate any virus-antibody aggregates, and the supernatant and pellet



Figs. 1–3. The anatomy and ultrastructure of the accessory salivary gland in *Metopolophium dirhodum*. **1,** Light micrograph of a 0.5-μm thick section stained with azure B showing a dorsal view of the accessory (ASG) and principal (PSG) salivary glands in relation to the salivary duct (SD), subesophageal nerve ganglion (SN), optic lobe (OL), and eye (E) on the left side of the aphid head. M = Muscle; bar = 100 μm. **2,** Nomarski interference contrast light micrograph of the accessory salivary gland. Three of four secretory cells (1–3) are visible. Microvilli-lined canals (unlabeled arrows) are visible throughout the cytoplasm. The lumen of each canal opens directly into the salivary duct (SD). Note the orientation of the basal lamina (BL) to the hemocoel (H) or body cavity and the orientation of the gland to adjacent nerve tissue of the optic lobe (OL) and subesophageal nerve ganglion (SN) and to adjacent muscle tissue (M). N = Nucleus; bar = 20 μm. **3,** Transmission electron micrograph showing the ultrastructure of an accessory salivary gland secretory cell. The gland is surrounded by an extracellular basal lamina (BL) secreted by the cells. Basal plasmalemma invaginations (IN) are associated with concentrations of mitochondria (M). Rough endoplasmic reticulum (RER) is distributed evenly throughout the cytoplasm. Below the area of membrane invaginations are concentrated lysosomal vesicles (L) and osmophilic (OV), crystalline (CSV), and amorphous (AV) secretory vesicles. The cell apical plasmalemma (APL) invaginates to form microvilli-lined canals (C) that branch throughout the cytoplasm and connect to the salivary duct. BPL = Basal plasmalemma; bar = 2 μm.


SN
BL-1
BL-2
BPL
M
RER
ASG
4
H
BL
BPL
R
CP
5
TV
C
V
APL
6
M
TV
CV
7
R
8
MV
C
C
V
APL
9
C
CP
CV
10

separated. Pellets were then resuspended in 0.01 M potassium phosphate buffer (pH 7.0). Formvar-carbon-coated 300-mesh EM grids were then floated on drops of each supernatant or pellet sample for 10 min, rinsed briefly in water, and then stained for 3 min in 2% aqueous uranyl acetate. Grids were examined by EM for virus aggregation and integrity as determined by particle diameter and staining characteristics. Samples treated as above were incubated for 30 min on EM grids coated with antirabbit or antimouse antibodies to verify antibody and Fab fragment binding to nonaggregated virions. To test for antibody-virion integrity following injection into aphids, aphids were injected with MAV (50 μg/ml) or with various MAV-antibody mixtures as described above. Injected aphids were allowed to feed on oats for 40 h, then homogenized individually in 20 μl of 10 mM potassium phosphate buffer (pH 7.0), and observed for intact virions in the homogenate by use of antibody-coated grids and immunospecific electron microscopy (ISEM) as described (1).

Electron microscopy. For ultrastructural examination, aphids were briefly dipped into 50% ethanol to aid wetting, immediately rinsed in distilled water, and submerged in a drop of fixative (1% formaldehyde, 2% glutaraldehyde, 0.01% CaCl, and 0.05% sodium azide in 0.02 M sodium cacodylate buffer, pH 7.4) supported on a plastic petri dish on the stage of a dissecting microscope. While submerged in fixative, the aphids were bisected with a razor blade through the middle of the thorax. The aphid tissues were transferred to a vial containing fresh fixative and were fixed overnight at 4 C. The aphids were then rinsed in 0.1 M cacodylate buffer, postfixed for 1 h in 2% osmium tetroxide in 0.1 M buffer, rinsed in buffer and water, incubated for 1–2 h in 2% aqueous uranyl acetate, dehydrated in an acetone series, and infiltrated with an Epon-Araldite formula plastic (Electron Microscopy Sciences, Fort Washington, PA) over 2 days at room temperature. Blocks were hardened at 60 C over 24 h. Sections 0.25 μm thick were prepared from the head and thorax region of each aphid, stained with azure B, and examined by interference contrast light optics to identify accessory salivary glands. Salivary glands were ultrathin sectioned at 60-nm thickness and contrasted with 2% uranyl acetate and 0.4% lead citrate prior to examination on a Joel 1200 transmission EM at the Electron Microscope Facility for the Life Sciences in the Biotechnology Institute at Penn State University. Generally, two or three sections on each of two grids were examined for each sample.

RESULTS

Transmission pathway through vectors. The internal anatomy and salivary structure of the five cereal aphid species examined were similar to those of *M. dirhodum* shown in Figures 1–3. In a dorsal view, the accessory salivary gland was observed anterior to the principal salivary gland, lateral to the subesophageal nerve ganglion, and posterior to the optic lobe (Fig. 1). A pair of accessory and principal salivary glands occurred on each side of the aphid's head. The size of the accessory gland varied widely with the size of the aphid but was approximately 50–100 μm in diameter. The accessory salivary glands of the cereal aphids consisted of four wedge-shaped secretory cells connected at their apical ends to the salivary ducts (Fig. 2). When thick sections were viewed by interference contrast optics, the cytoplasm appeared filled with vesicles and granules. Dark-staining cytoplasmic granules 1–3 μm in diameter bordered the cell membrane (basal plasmalemma) facing the hemocoel. Segments of microvilli-lined canals with characteristic darkly stained central regions could be seen throughout the cytoplasm. The four nuclei of the accessory gland were much larger than the nuclei of adjacent cell types and ranged from 10–20 μm in diameter. When observed by EM (Fig. 3), the accessory gland was seen to be surrounded by a continuous layer of basal lamina. The dark-staining granules were observed to be mitochondria concentrated among the numerous invaginations of the basal plasmalemma. Below the membrane invaginations were concentrated a variety of vesicles that contained crystalline, osmophilic, or amorphous contents. Below the concentration of vesicles were seen branches of the microvilli-lined canal formed by the invagination of the apical plasmalemma. The lumen of this canal from each secretory cell opens directly into the chitin-lined salivary duct. The salivary duct leading out of the accessory gland joins a short salivary duct draining the principal gland to form a common salivary duct that leads through a salivary pump to the salivary canal in the stylets (15).

When *S. avenae* were injected with 0.5 ng of MAV virions per aphid, virions were consistently observed embedded in the basal lamina surrounding the accessory salivary gland (Fig. 4) but were not associated with basal lamina of the subesophageal nerve ganglion, principal salivary gland, or adjacent muscle and connective tissue. Virions closely associated with the plasmalemma were observed concentrated in linear arrangements within the lumen of plasmalemma invaginations. Virions were commonly observed within coated pits associated with the basal plasmalemma apparently in the process of invaginating into the cytoplasm by endocytosis (Fig. 5). Virions could be differentiated from cytoplasmic ribosomes by size and ultrastructural detail at high magnifications and by differential susceptibility to RNase digestion (3). On the basis of these criteria, no virions were observed free in the cytoplasm. Within the cytoplasm, virions were typically observed in elongated tubular vesicles (Fig. 6) below the membrane invaginations, in tubular vesicles adjacent to the microvilli-lined canals, and in coated vesicles (Figs. 7 and 8). Characteristic clathrin protein spikes radiating from membrane vesicles clearly identified virion-containing coated vesicles (Fig 8). The tubular vesicles were typically uncoated smooth membrane structures, except where coated pits were observed budding to form coated vesicles. Uncoated membrane vesicles containing virions were assumed to be cross sections of tubular vesicles and were infrequently observed. At the canal, virus particles occurred in coated pits attached to the canal membrane and in the canal lumen (Figs. 9 and 10). Virions were also observed in accessory salivary glands of *S. avenae* and *M. dirhodum* fed on MAV-infected oats but at reduced concentrations.

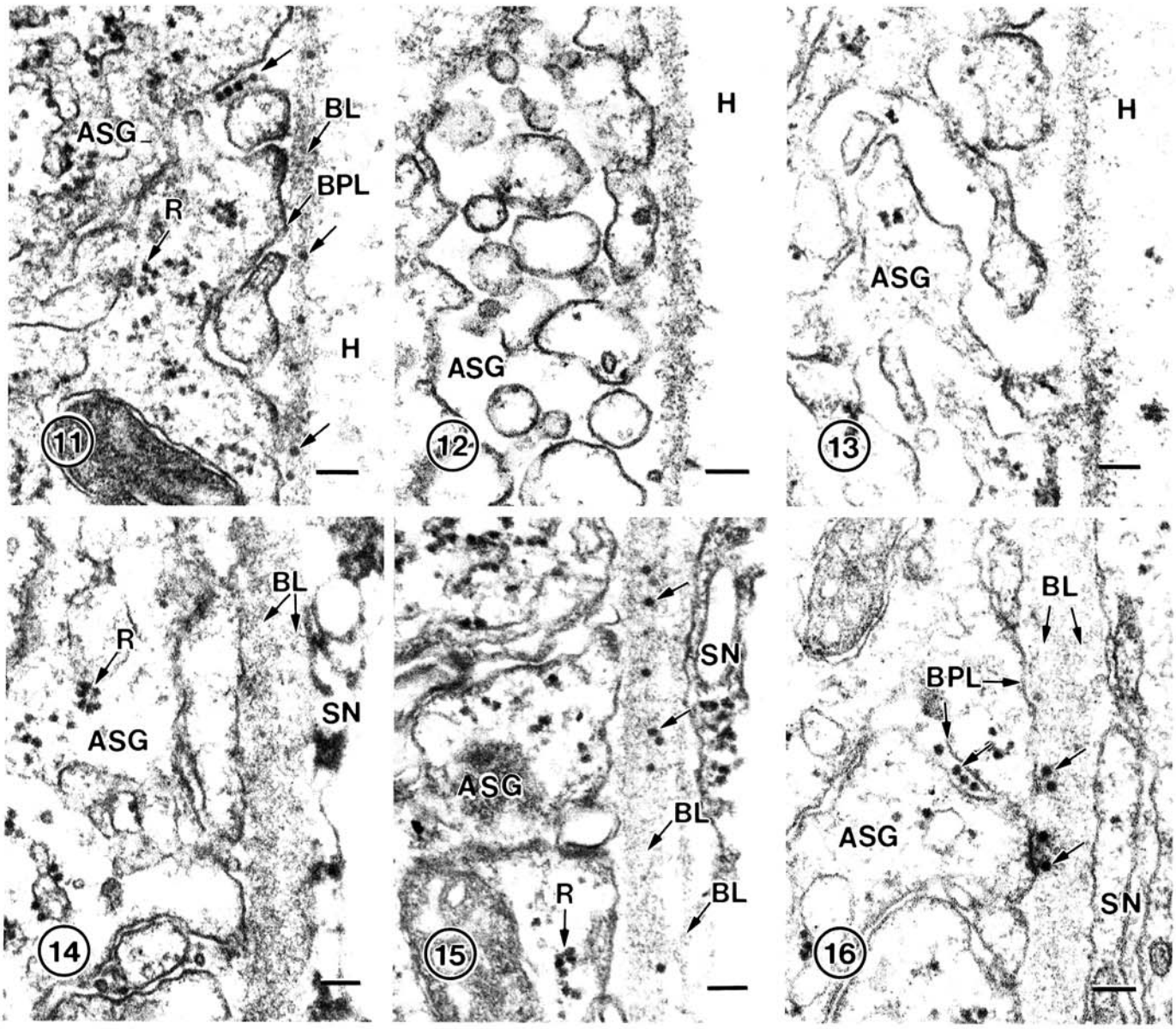
Transmission barriers. When *S. avenae* and *R. padi* were reared together on either MAV- or RPV-infected oats for 2 wk and then examined by EM for the presence of virions associated with the accessory salivary glands, virions of both MAV and RPV were observed in *S. avenae,* but only RPV was observed in *R. padi.* In *S. avenae,* virions were observed embedded in the basal lamina surrounding the accessory salivary gland and between the basal lamina and basal plasmalemma in 37 of 38 aphids fed on transmissible MAV and in 20 of 20 aphids fed on nontransmissible RPV. Only MAV virions, however, were observed intracellularly in the salivary gland, and only MAV was transmitted to plants. In *R. padi,* virions of the transmitted RPV were consistently observed embedded in the basal lamina and intracellularly in the

Figs. 4–10. Ultrastructure of membranes associated with transcellular transport of microinjected virions of the MAV isolate of barley yellow dwarf virus through the accessory salivary gland of *Sitobion avenae.* **4,** Virions of MAV embedded in the accessory salivary gland (ASG) basal lamina (BL-2) and concentrated in basal plasmalemma (BPL) invaginations (arrows). Note absence of virions in the basal lamina (BL-1) of the adjacent subesophageal nerve ganglion (SN). Particles in the cytoplasm are ribosomes associated with rough endoplasmic reticulum (RER). M = Mitochondria; bar = 500 nm. **5,** Virions penetrating the basal lamina (BL) from the hemocoel (H) and in a coated pit (CP) during endocytosis through the basal plasmalemma (BPL). Small irregularly shaped particles observed free in the cytoplasm are ribosomes (R), as determined by RNase digestion (3) and ultrastructure under higher magnification. **6,** Virions packaged in tubular vesicles (TV) adjacent to the apical plasmalemma (APL) lining a microvilli-lined canal (C). V = virion in canal. **7,** Virions in tubular vesicles (TV) and associated coated vesicles (CV). M = mitochondria. **8,** Comparison of a virion in a coated vesicle (arrow) to cytoplasmic ribosomes (R). **9,** A virion (V) being released from the accessory salivary gland cell into the canal lumen (C) by exocytosis through the apical plasmalemma (APL). MV = Microvilli. **10,** Virions (arrows) in the canal lumen (C) released from coated pits (CP) following fusion of coated vesicles (CV) with the cell membrane. Bars for Figures 5–10 = 100 nm

TABLE 1. Ability of MAV barley yellow dwarf luteovirus particles to penetrate the basal lamina surrounding the accessory salivary glands of five aphid species following microinjection of purified virions and a 24-h inoculation access period on Coastblack oats[a,b]

Experiment	*Sitobion avenae*	*Metopolophium dirhodum*	*Rhopalosiphum padi*	*Rhopalosiphum maidis*	*Schizaphis graminum*
1	5/5	...	0/5	...	...
2	5/5	...	1/5	...	...
3	3/3	...	0/3	0/3	0/3
4	3/3	...	0/3	0/3	0/3
5	6/7	6/7	2/7	0/7	1/7
Total	22/23	6/7	3/23	0/13	1/13
Percentage	96	86	13	0	8
Number transmitting[c]	59/74	1/12	3/61	0/50	0/39
Transmission percentage	80	8	5	0	0

[a]Individual third and fourth instar nymphs were microinjected with approximately 20 nl of density gradient purified MAV virions at concentrations of 50 (experiment 3), 75 (experiment 1), or 100 (experiments 2, 4, and 5) μg/ml suspended in 10 mM potassium phosphate buffer (pH 7.0). Aphids were then placed singly on 7-day-old oat seedlings and allowed to feed for 24 h. A subsample of 10–20 aphids was then removed from the plants and fixed for ultrastructural observation. The oats were then fumigated and observed in the greenhouse for symptoms over 6 wk.
[b]Number of aphids in which virus was observed in the basal lamina per total number of aphids examined from each species.
[c]Number of aphids transmitting MAV per total number of aphids tested.



Figs. 11–16. Affinity of the MAV isolate of barley yellow dwarf virus for accessory salivary gland basal lamina in aphids of five species 24 h after injection of purified MAV virions. Virions were consistently observed in **11,** basal lamina (BL) of *Sitobion avenae* and in **15,** *Metopolophium dirhodum* accessory salivary glands (ASG) but only rarely or not at all in **12,** *Rhopalosiphum padi*, **13,** *R. maidis*, and **14,** *Schizaphis graminum*. **15,** In *M. dirhodum*, virions were observed embedded in the outer layers of the basal lamina but not adjacent to the basal plasmalemma (BPL) or within cytoplasmic vesicles. **16,** In *S. avenae*, virions (arrows) always were observed having penetrated the basal lamina. In Figure 16, note the affinity of MAV virions for the salivary basal lamina but their absence from the adjacent basal lamina of the subesophageal nerve ganglion (SN). H = hemocoel; R = ribosomes; bars = 100 nm.

salivary gland of all 31 aphids examined. Virions of nontransmitted MAV, however, were never observed in any of 50 *R. padi* examined from a total of five separate experiments in which different plants were used as virus sources. No virions were observed in any of 10 aphids of each species reared on healthy oats as controls.

In a subsequent series of experiments, individual third instar nymphs of *M. dirhodum, R. maidis, R. padi, S. graminum,* and *S. avenae* were injected with 0.5, 1.0, or 2.0 ng of purified MAV virions, allowed a 24-h IAP on oats, and then fixed for EM observation. When accessory salivary glands of aphids of each species from five experiments were examined, virions of MAV were consistently visualized embedded in the basal lamina of *S. avenae* and *M. dirhodum* but only rarely in *R. padi* and *S. graminum* and never in *R. maidis* (Table 1). No MAV transmission occurred by *R. maidis* or *S. graminum;* and *R. padi* transmitted MAV to only three of 61 plants when injected with 2 ng of purified virions but did not transmit MAV when injected with 0.5 or 1.0 ng of virions. The Cornell clone of *M. dirhodum* used in these experiments was a poor vector of MAV when injected with purified MAV; however, MAV was consistently observed in the salivary basal lamina of this species.

Three types of MAV virion-salivary basal lamina interactions were identified. 1) In the primary vector, *S. avenae* (Fig. 11), virions were typically observed throughout the basal lamina, in the space between the basal lamina and the basal plasmalemma, and within plasmalemma invaginations into the cytoplasm. 2) In *R. padi* (Fig. 12), *R. maidis* (Fig. 13), or *S. graminum* (Fig. 14), virions were almost never observed in the salivary basal lamina and were never observed free in the hemocoel or attached to the outer edge of the salivary gland basal lamina. 3) In *M. dirhodum,* MAV was primarily observed embedded only in the outer half of the salivary basal lamina facing the hemocoel (Fig. 15) and was rarely observed to have penetrated the basal lamina. Specificity of MAV for salivary gland basal lamina of *S. avenae* and *M. dirhodum* is illustrated in Figures 15 and 16, in which virions can be easily observed in salivary basal lamina but not in basal lamina of the adjacent subesophageal nerve ganglion. Similar views were observed with other adjacent tissues (not shown).

Although injected MAV virions were detected in the Cornell colonies of *M. dirhodum,* few aphids (8%) transmitted the virus (Table 1). Data from observations of individual aphids injected with 2 ng of MAV (Table 2) indicated that MAV remained in the basal lamina and only rarely was transported into and through the salivary gland in *M. dirhodum,* as indicated by the one transmission. In addition, virus density observed in the basal lamina of *M. dirhodum* was much less than that of *S. avenae* injected with the same virus preparation. Virions in basal lamina of nonvector species occurred only rarely in a few individuals. To further examine the MAV-basal lamina interaction in *M. dirhodum,* the inefficient MAV-transmitting Cornell colony of *M. dirhodum* was compared to an efficiently transmitting *M. dirhodum* colony maintained at Penn State. Both of these separately maintained colonies were initiated in 1983 from the same California clone of *M. dirhodum* collected near Davis in 1981 (4). When individual aphids of both colonies were injected with 1 ng of MAV, fed 24 h on oat seedlings, fixed for EM, and observed for the presence of the virus in the salivary basal lamina, differences were observed that corresponded to transmission efficiency. The efficiency of MAV transmission by single aphids from the Cornell and Penn State colonies following MAV injection was 15 and 65%, respectively. Five aphids were examined by EM for each treatment in each of two replicated experiments. In the Cornell *M. dirhodum,* virions were observed in the salivary basal lamina in six of 10 aphids and intracellularly in only one of 10 aphids. In the Penn State *M. dirhodum,* virions were observed penetrating basal lamina in 10 of 10 aphids and intracellularly in seven of 10 aphids. In addition, the density of virions embedded in the basal lamina of individual aphids from the Cornell and Penn State colonies averaged 8 ± 7 and 60 ± 20 virions per 10 μm of basal lamina length (eight or 60 virions per 0.05 μm^3, assuming a basal lamina width of 75 nm and a section thickness of 70 nm), respectively. These results indicated differences in basal lamina that affected virus transmission between colonies of the same aphid species.

To further test for a basal lamina penetration barrier to the accessory salivary gland, we injected approximately 30 ng of purified brome mosaic virions (BMV) into each of 30 *R. padi,* allowed them a 24-h IAP on oat seedlings, and then observed the salivary gland basal lamina for BMV penetration. Brome mosaic virus is not known to be transmitted by *R. padi* in nature (13). When the salivary glands of 10 injected aphids were examined by EM, high concentrations of BMV particles were observed in the hemocoel adjacent to the salivary gland and lining the salivary basal lamina in all 10 aphids examined. However, no virions were observed penetrating the basal lamina. None of the 30 injected aphids transmitted BMV. These observations supported the idea that the basal lamina selectively regulated virus movement into the salivary gland.

Antibody neutralization of transmission. A series of experiments was done to determine how the binding of monoclonal

TABLE 2. Localization of virions of MAV barley yellow dwarf luteovirus in basal lamina (BL) and intracellularly (IC) in accessory salivary glands in five aphid species following microinjection of purified virions of MAV into the aphid hemocoel and a 24-h inoculation access period on Coastblack oats[a,b]

	Sitobion avenae		*Metopolophium dirhodum*		*Rhopalosiphum padi*		*Rhopalosiphum maidis*		*Schizaphis graminum*	
Aphid	BL	IC	BL	IC	BL	IC	BL	IC	BL	IC
1	−	−	+	−	−	−	−	−	−	−
2	+	+	+	−	−	−	−	−	−	−
3	+	+	+	−	−	−	−	−	(+)	(+)
4	+	+	+	−	(+)	(+)	−	−	−	−
5	+	+	+	−	(+)	(+)	−	−	−	−
6	+	+	+	−	−	−	−	−	−	−
7	+	+	+	−	−	−	−	−	−	−
Density[c]	47		28		<1		0		<1	
Transmission[d]	9/12		1/12		2/10		0/12		0/11	

[a]Third and fourth instar nymphs were injected with approximately 20 nl of 100 μg/ml of density gradient purified MAV virions (2 ng per aphid) suspended in 10 mM phosphate buffer (pH 7). Aphids were then placed individually on 7-day-old oat seedlings for a 24-h inoculation feeding. The aphids were then fixed for electron microscopy, and the plants were fumigated and observed for symptoms over 6 wk.

[b]The perimeter of the basal lamina around the accessory gland was scanned for virions in a minimum of two sections on two grids made from each aphid. The salivary cell cytoplasm in each section was also scanned for the presence of virions in plasmalemma invaginations, in coated and tubular vesicles, and in the canal lumen. + = Virions were consistently observed in the location indicated; (+) = at least one obvious virion was observed but virions were very rare; and − = no virions were observed.

[c]Average number of virions per 10 μm of basal lamina length (or 0.05 μm^3 of basal lamina, assuming a basal lamina width of 75 nm and a section thickness of 70 nm). Counts based only on those aphids in which virus was identified.

[d]Number of aphids transmitting MAV per total number tested from each treatment.

antibodies to specific epitopes of the luteovirus capsid protein would interfere with virus recognition, attachment, and penetration of the accessory salivary gland. Results (Table 3) indicated that all homologous anti-MAV antibody treatments greatly reduced or eliminated MAV transmission relative to the buffer or anti-RPV controls. When MAV-antibody suspensions were examined, the whole anti-MAV IgG was found to aggregate the virus as expected. Virus incubated with anti-MAV Fab fragments, however, did not aggregate and appeared similar to virions incubated with anti-RPV or buffer. In addition, we know that the Fab treatments did not grossly damage the MAV virions because normal-appearing, intact particles were observed after 3 and 7 days of incubation in vitro and because intact MAV-Fab particles were recovered from aphid hemolymph 24 h after the initial injection and identified by ISEM on antimouse antibody-coated grids. When the salivary glands of injected aphids were examined by EM, MAV virions in buffer or those treated with anti-RPV were consistently observed embedded in the salivary basal lamina and intracellularly in the salivary gland. In contrast, virions of MAV incubated with any of the anti-MAV whole antibody or Fab preparations were never observed in the basal lamina of the aphids examined. These results suggest that alteration of the virion capsid by attachment of antibody Fab fragments interfered with the ability of virions to penetrate basal lamina.

DISCUSSION

Results of this study support the hypothesis that receptor-mediated endocytosis is the mechanism involved in luteovirus recognition and transport into the accessory salivary gland. Previous studies (5) involving competitive inhibition of transmission by serologically similar luteoviruses have indirectly implicated such a mechanism. However, ultrastructural images supporting this hypothesis have been limited (6). When aphids acquire luteoviruses by feeding on infected plants, virus density at the salivary gland is relatively low, and virions associated with the basal plasmalemma are only rarely observed in membrane structures suggesting endocytosis (3). In this study, however, the high concentration of virus injected into the hemocoel allowed us to consistently observe virions attached to the salivary basal plasmalemma and within coated pits in the process of being transported into the cytoplasm by endocytosis.

The ability of MAV to penetrate the accessory salivary gland basal lamina of only specific aphid species indicates a role for the basal lamina in determining vector-specific transmission. On the bases of our ultrastructural observations and transmission studies, at least three types of virus-basal lamina interactions occurred (Fig. 17). In the first type of interaction (nonpenetrating-nontransmitted, type A), virions of MAV did not attach to the basal lamina of some nonvector aphid species. In the second type of interaction (penetrating-nontransmitted, type B), virions were able to penetrate the basal lamina and were either trapped in the basal lamina or were prevented from entering the salivary gland at the cell membrane. This type of interaction has been previously observed for the RPV-NY isolate in *S. avenae* (6). The third type of interaction (penetrating-transmitted, type C), occurs for all transmissible luteovirus-aphid combinations studied to date. In this type, the virus penetrates the basal lamina and is recognized and transported through the cell membrane into the salivary gland.

Although many insect-transmitted viruses must pass through basal lamina surrounding several insect tissues, we are unaware of studies to explain this process. Previous studies have identified the basal lamina as a barrier that prevents the passage of molecules larger than 15 nm (7,17). This idea is not compatible, however, with observations that luteoviruses 25 nm in diameter pass through gut and salivary gland basal lamina. Current concepts suggest that basal laminae are elastic structures that vary in composition and function among different tissues within an organism (22). Basal laminae are constructed of two major glycoproteins, collagen and laminin, that self-assemble into irregular three-dimensional networks forming a meshlike covering over the cell surface. A variety of other molecules associate with the collagen and laminin networks that contribute to the functional differences among basal laminae of various tissues (2). Although the function of most of these compounds is unknown, their structural relationships to other regulatory molecules suggest that they also may possess regulatory functions.

The above information suggests that the aphid salivary gland basal lamina may have the molecular complexity necessary to allow species-specific regulation of virus recognition and penetration. This is consistent with our observation that the luteoviruses specifically attached to and penetrated only basal lamina of the accessory salivary gland. In addition, most virions penetrated the basal lamina only at the anterior end of the gland and were observed with decreasing frequency from lateral to posterior margins of the gland. Because the aphids were injected with high concentrations of virus, all areas around the circumference of the gland would have been equally exposed to virus. Differential binding to and penetration of the basal lamina by viruses suggests fundamental differences in basal lamina structure in different parts of the gland and among different tissues. How 25-nm virions are able to diffuse or be moved through the dense meshwork of basal lamina is unknown. Perhaps surface characteristics of virus capsid proteins are recognized by basal lamina components that allow the virion to come into contact with the collagen-laminin network and penetrate the basal lamina. According to such a hypothesis, both virus and basal lamina structure would influence this interaction and dictate the efficiency of penetration. If virions lacked appropriate surface structure, then in some cases the particles might not be able to make the initial

TABLE 3. Transmission and visualization of barley yellow dwarf virions in basal lamina surrounding the accessory salivary glands of *Sitobion avenae* microinjected with monoclonal antibody-treated MAV prior to a 24-h inoculation access period on 7-day-old Coastblack oats

Antibody treatment[a]	Virus observed in basal lamina[b]	Virus density in basal lamina[c]	Transmission	
			Number[d]	Percentage
None	12/12	65	31/35	89
RPV-1	10/10	75	28/41	68
MAV-1	0/9	0	0/16	0
MAV-1 Fab	1/5	<1	1/12	8
MAV-2	0/5	0	2/10	10
MAV-4	0/15	0	1/39	3
MAV-4 Fab	0/10	0	7/40	18

[a]Purified MAV virions were diluted to 25 μg/ml in 10 mM phosphate buffer containing 100 μg/ml of either the whole monoclonal antibody or Fab fragment indicated and incubated about 1 h at room temperature. No aggregation of virus by RPV-1 or Fab fragments was detected. Third and fourth instar nymphs were then microinjected with approximately 20 nl of MAV-antibody suspension and given a 24-h feeding on oats. A subsample of aphids was then fixed for electron microscopy. All plants were fumigated and observed for 6 wk for symptoms indicative of transmission.
[b]Number of aphids in which virus was observed per total number of aphids examined.
[c]Average number of virions per 10 μm of basal lamina length determined from five aphids randomly selected from each treatment. Approximate volume of 10 μm of basal lamina is 0.05 μm^3, assuming a basal lamina width of 75 nm and a section thickness of 70 nm.
[d]Number of aphids transmitting MAV per total number of aphids tested in each treatment.

attachment to the basal lamina. In other virus-vector combinations, virus might even be repelled because of equivalent net surface charges of the virions and the basal lamina acting as a charge-dependent biological filter (7,22). Different aphid species with salivary basal lamina of slightly differing composition would each respond differently to viruses with different surface properties.

A mechanism for virus-basal lamina recognition is consistent with our observation of subtle differences in MAV penetration of salivary basal lamina in *M. dirhodum* from two different colonies, which differed in transmission efficiency, and suggests that these aphids may differ slightly in basal lamina composition or structure. The importance of virus capsid surface structure for basal lamina recognition is supported by the reduced ability of virions coated with antibody Fab fragments to attach to the salivary basal lamina outer surface (Table 3). Apparently, the antibody peptides attached to the virion surface prevented important initial interactions with the basal lamina. Unfortunately, this result means that monoclonal Fab fragments to virus cannot be used in studies of capsid-salivary membrane interactions. The specificity and consistency of basal lamina selectivity was further demonstrated by the inability of BMV to penetrate the salivary basal lamina of *R. padi* even when injected at 15 times the concentration of the highest luteovirus injection. Although BMV filled the hemocoel adjacent to the salivary gland, no virions were observed attached to or penetrating the basal lamina.

In summary, we have presented evidence that the extracellular basal lamina surrounding the accessory salivary gland functions as a selective barrier regulating vector-specific transmission of luteoviruses. The virus-basal lamina interactions involved in virus penetration of the salivary gland are unknown, but evidence suggests that virus protein recognition and interaction with basal lamina glycoprotein may be important. Salivary basal lamina selectivity that controls transmission of luteoviruses in the vector is believed to occur in addition to the virus-membrane interactions at the hindgut apical plasmalemma (4) and at the salivary basal plasmalemma (6). On the basis of these observations, the circulative route of BYDV through aphid vectors is believed to involve the following stages. Virions are initially taken into the aphid stylet food canal when the aphid feeds on luteovirus-infected phloem cells. Ingested virions then move suspended in sap through the lumen of the alimentary canal until they reach the region of the hindgut. At the hindgut, virions may attach to the apical plasmalemma, initiating endocytosis of the virions into the epithelial cells forming the gut wall (3). Virions that do not come in contact with the cell membrane or virions that are not recognized by virus-specific receptors on the membrane continue to move through the alimentary canal and are voided in the honeydew (4). Endocytosed virions are transported in coated vesicles to endosomal vesicles or receptosomes and are then packaged into tubular vesicles that move to the basal plasmalemma and release the virions into the hemocoel by exocytosis. All luteoviruses studied to date have been observed to move freely through the basal lamina surrounding the gut and into the hemocoel. Virions suspended in the hemolymph diffuse throughout the aphid's body. To be transmitted, virions must pass through the accessory salivary gland and be excreted with salivary secretions during aphid feeding (3). In order to reach the accessory salivary gland, virions must first be able to penetrate the extracellular basal lamina surround-

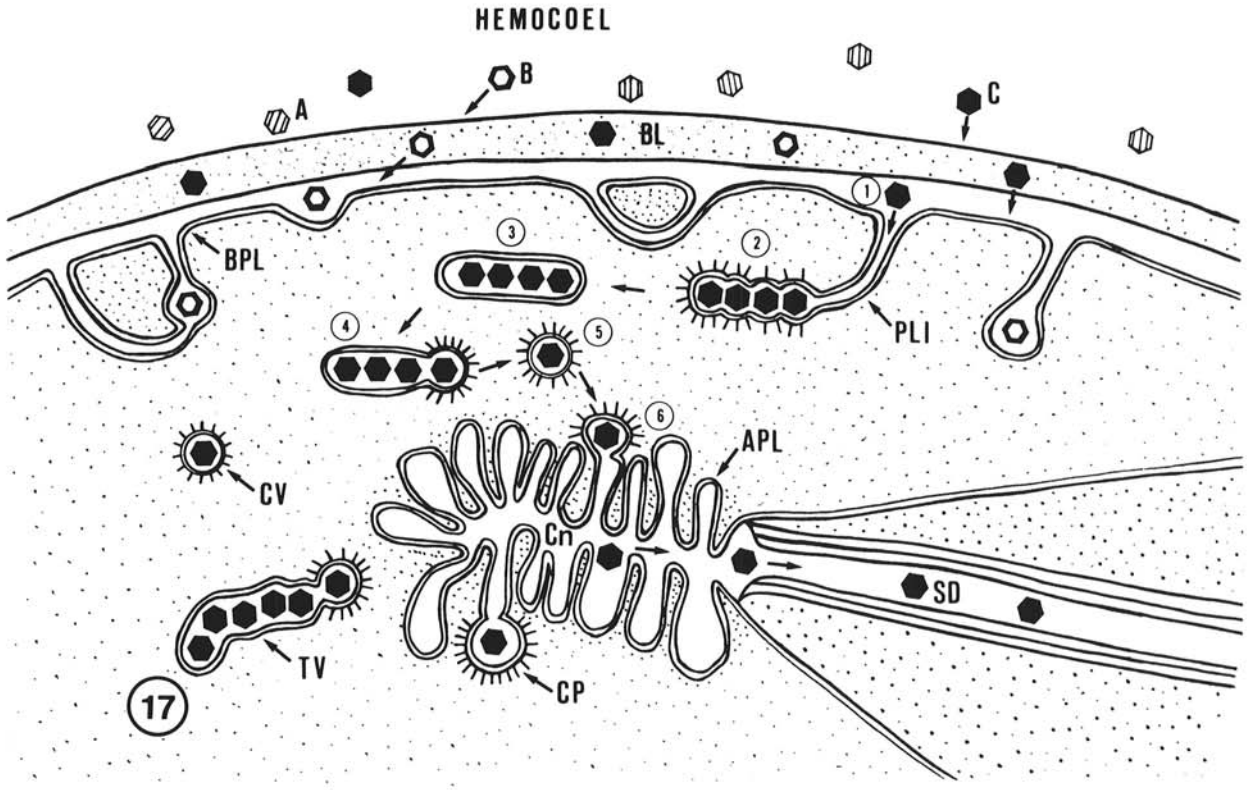


Fig. 17. The interactions of luteoviruses with the accessory salivary gland of aphid vector species. Three types of interactions were observed when virions of the MAV isolate of barley yellow dwarf virus were acquired by aphids that fed on infected plants or that were injected with purified virions into the hemocoel. In the first type of interaction, MAV virions had no affinity for the salivary basal lamina (BL) of specific aphid species and did not attach to or penetrate the basal lamina (A = nonpenetrating-nontransmitted virions). In other species, MAV virions did exhibit affinity for the salivary basal lamina and were able to attach to and, in some cases, were able to penetrate the basal lamina. However, these virions were unable to initiate endocytosis at the basal plasmalemma (BPL) and were not transmitted (B = penetrating-nontransmitted virions). In the third type of interaction, virions consistently penetrated the basal lamina (1), were aggregated in plasmalemma invaginations (PLI), and were endocytosed into the cell by coated pit formation (2). Virions acquired into the cytoplasm accumulated at the apical end of the cell in tubular vesicles (3). Individual virions budded from the tubular vesicles by coated pit formation (4) and were transported to the salivary canal (Cn) in coated vesicles (5) that fused to the apical plasmalemma (APL), releasing the virion into the canal lumen (6). Transcytosed virions were then able to move into the salivary duct (SD) (C = penetrating-transmitted virions). TV = tubular vesicle; CP = coated pit; and CV = coated vesicle.

ing the gland. Virions unable to penetrate this barrier are retained in the hemocoel and cannot be transmitted. Some luteoviruses may penetrate the basal lamina very slowly or only occasionally, and these viruses may be inefficiently transmitted. Other luteoviruses appear to concentrate in the basal lamina of specific aphid vector species and readily diffuse through to the salivary gland basal plasmalemma. Virions of nontransmissible luteoviruses able to penetrate the basal lamina are not recognized at the basal plasmalemma and accumulate in the space between the basal lamina and cell membrane (6). Transmissible luteoviruses are recognized at the basal plasmalemma, attach to the membrane, are endocytosed into the cell, and accumulate in tubular vesicles adjacent to the apical plasmalemma making up the salivary canal. Coated vesicles containing individual virions bud off the tubular vesicles and transport the virions to the apical plasmalemma where the virions are released by endocytosis into the salivary canal lumen. These virions are now structurally external to aphid tissues and are free to move with salivary secretions out of the aphid and through the stylets into new plant hosts during aphid phloem feeding.

LITERATURE CITED

1. Derrick, K. S. 1973. Quantitative assay for plant viruses using serologically specific electron microscopy. Virology 56:652-653.
2. Fessler, J. H., and Fessler, L. I. 1989. Drosophila extracellular matrix. Annu. Rev. Cell Biol. 5:309-339.
3. Gildow, F. E. 1987. Virus-membrane interactions involved in circulative transmission of luteoviruses by aphids. Curr. Top. Vector Res. 4:93-120.
4. Gildow, F. E. 1993. Evidence for receptor-mediated endocytosis regulating luteovirus acquisition by aphids. Phytopathology 83:270-277.
5. Gildow, F. E., and Rochow, W. F. 1980. Importance of capsid integrity for interference between two isolates of barley yellow dwarf virus in an aphid. Phytopathology 70:1013-1015.
6. Gildow, F. E., and Rochow, W. F. 1980. Role of accessory salivary glands in aphid transmission of barley yellow dwarf virus. Virology 104:97-108.
7. Giorgi, F., Yin, C.-M., and Stoffolano, J. G., Jr. 1991. Permeability barriers and anionic sites of the ovarian basal laminae in the black blowfly *Phormia regina* (Meigen) (Diptera, Calliphoridae). Invertebr. Reprod. Dev. 19:37-44.
8. Gray, S. M. 1991. Monoclonal antibodies against barley yellow dwarf virus inhibit circulative transmission at different sites within aphid vectors. (Abstr.) Phytopathology 78:1538.
9. Gray, S. M., Power, A. G., Smith, D. M., Seaman, A. J., and Altman, N. S. 1991. Aphid transmission of barley yellow dwarf virus: Acquisition access periods and virus concentration requirements. Phytopathology 81:539-545.
10. Harris, K. F., Bath, J. E., Thottappilly, G., and Hooper, G. R. 1975. Fate of pea enation mosaic virus in PEMV-injected pea aphids. Virology 65:148-162.
11. Hsu, H. T., Aebig, J., and Rochow, W. F. 1984. Differences among monoclonal antibodies to barley yellow dwarf viruses. Phytopathology 74:600-605.
12. Jedlinski, H. 1981. Rice root aphid, *Rhopalosiphum rufiabdominalis,* a vector of barley yellow dwarf virus in Illinois, and the disease complex. Plant Dis. 65:975-978.
13. Lane, L. C. 1977. Brome mosaic virus. No. 180 in: Descriptions of Plant Viruses. Commonw. Mycol. Inst./Assoc. Appl. Biol., Kew, England.
14. Martin, R. R., Keese, P. K., Young, M. J., Waterhouse, P. M., and Gerlach, W. L. 1990. Evolution and molecular biology of luteoviruses. Annu. Rev. Phytopathol. 28:341-363.
15. Ponsen, M. B. 1977. Anatomy of an aphid vector: *Myzus persicae.* Pages 63-82 in: Aphids as Virus Vectors. K. F. Harris and K. Maramorosch, eds. Academic Press, New York.
16. Power, A. G., Seaman, A. J., and Gray, S. M. 1991. Aphid transmission of barley yellow dwarf virus: Inoculation access periods and epidemiological implications. Phytopathology 81:545-548.
17. Reddy, J. T., and Locke, M. 1990. The size limited penetration of gold particles through insect basal laminae. J. Insect Physiol. 36:397-407.
18. Rochow, W. F. 1969. Biological properties of four isolates of barley yellow dwarf virus. Phytopathology 59:1580-1589.
19. Rochow, W. F., and Carmichael, L. E. 1979. Specificity among barley yellow dwarf viruses in enzyme immunosorbent assays. Virology 95:415-420.
20. Rochow, W. F., and Duffus, J. E. 1981. Luteoviruses and yellows diseases. Pages 147-170 in: Handbook of Plant Virus Infections and Comparative Diagnosis. E. Kurstak, ed. Elsevier North-Holland Biomedical Press, Amsterdam.
21. Waterhouse, P. M., Gildow, F. E., and Johnstone, G. R. 1987. The luteovirus group. No. 339 in: Descriptions of Plant Viruses. Commonw. Mycol. Inst./Assoc. Appl. Biol., Kew, England.
22. Yurchenco, P. D., and Schittny, J. C. 1990. Molecular architecture of basement membranes. FASEB J. 4:1577-1590.