Some Cucumber Mosaic Virus Antisera Contain Antibodies Specific for Both Peanut Stunt Virus and Chrysanthemum Mild Mottle Virus

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ABSTRACT

Antibodies in ascites fluids prepared against a legume strain of cucumber mosaic virus (CMV-LE), peanut stunt virus (PSV), or chrysanthemum mild mottle virus (CMMV), reacted only with their respective homologous antigens in both ring-interface and gel-diffusion tests. There was no indication that any two of the three viruses were related serologically. However, five rabbit antisera that had been prepared earlier against two other CMV strains (CMV-O and CMV-Y) reacted with PSV and CMMV in addition to CMV. Gel-diffusion tests and ring interface tests made with these five antisera after absorption with individual viruses indicated that the antibodies to each virus present in each antisera were separate and distinct. Both PSV and CMMV were detected serologically in two CMV isolates, suggesting that antisera which contain antibody mixtures likely resulted from the use of mixed virus isolates. Mixed CMV antisera appear to be common and may account for the so-called "broad spectrum" and "narrow spectrum" antisera previously used to determine relationships in this group of small isometric viruses.

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Cucumber mosaic virus (CMV), tomato aspermy virus (TAV) and peanut stunt virus (PSV) are small isometric viruses that have similar physical and chemical properties (10). Despite their many similarities, attempts to determine serological relationships among these viruses have produced conflicting reports (7). Some strains of CMV and TAV are reported to be related serologically, whereas other strains of both viruses show no such relationship (3). Lawson (7) demonstrated that one reason for these anomalous results was the choice of the aspermy-type virus used. One aspermy-type virus isolated from chrysanthemum not only differed serologically from CMV but also differed from the TAV originally found in tomato (1). A second reason for some of the conflicting reports, seems to be the choice of antisera used for tests. "Broad spectrum" antisera against TAV and some CMV strains have been found to react with heterologous viruses in tests where "Narrow spectrum" antisera do not (3). This variability among antisera is further complicated by the fact that some CMV antisera from America and Australia also contain antibodies specific for PSV in addition to those specific for CMV (9).

In preliminary studies on the serological relationships among three small isometric viruses isolated in Japan, we found that some Japanese CMV antisera reacted not only with CMV but also with PSV and with chrysanthemum mild mottle virus (CMMV). This latter is an aspermy-type virus previously shown to be unrelated serologically to CMV (13). These results prompted us to test certain CMV antisera both qualitatively and quantitatively to determine whether different antibodies were present for each virus, or whether some of the CMV-specific antibodies were also capable of reaction with other viruses. In addition, we tested selected CMV isolates serologically for the presence of PSV and CMMV.

MATERIALS AND METHODS.—*Antibody preparation.*—The CMV antisera we found to contain antibodies against PSV and CMMV had been prepared in rabbits 1-2 years earlier using preparations from infected *Nicotiana tabacum* L. 'Ky 57' leaves partially purified by

TABLE 1. Reciprocal titer of antibodies present in various antisera and ascites fluids. CCMV = chrysanthemum mild mottle virus. PSV = peanut stunt virus. CMV = cucumber mosaic virus.

Ascites fluid or antiserum	Test antigen[a]					
	Healthy[b]	CCMV	PSV J	PSV K61	CMV O[c]	CMV Az
Ascites Fluid						
Control	64	0	0	0	0	0
Healthy	128	0	0	0	0	0
CMMV	64	2,048	0	0	0	0
PSV-J	64	0	1,024	1,024	0	0
PSV-K61	64	0	1,024	1,024	0	0
CMV-LE	64	0	0	0	2,048	2,048
Antiserum						
PSV-J	0	0	64	64	0	0
PSV-W	0	0	64	64	0	0
CMV-OA[d]	128[e]	16[f]	16[f]	16[f]	2,048	NT[g]
CMV-OA2[d]	256[e]	16[f]	16[f]	16[f]	4,096	NT
CMV-OFA[d]	512[e]	16[f]	16[f]	16[f]	4,096	NT
CMV-YFA[d]	8[e]	32[f]	128	128	1,024	1,024
CMV-YFI[d]	16[e]	8[f]	512	512	8,192	8,192

[a]Virus concentration = 100 μg/ml.
[b]The concentration of healthy components was not determined.
[c]Although CMV-O antisera and CMV-O test antigen are listed in this table it should be recognized that nearly two years elapsed between antiserum preparation and these tests. During this period the stock isolate was subcultured numerous times.
[d]A = antigen was emulsified with complete adjuvant before intramuscular injection. A2 = two adjuvant injections. F = antigen fixed with formalin. I = interperitoneal injection.
[e]Healthy antibodies were removed by absorbing with healthy antigens without affecting the titer of the virus-specific antibodies.
[f]Tests were made with antisera previously absorbed with healthy antigen.
[g]Not tested.

Scott's procedure (11). Because substantial amounts of normal plant components remain after this treatment, the exact amount of virus used in each injection was not known. One or two intramuscular injections were made with antigens emulsified in Freund's complete adjuvant. The exact protocol used for preparation of each antiserum is listed in Table 1. Also, prior to this study, antibodies against a legume strain of CMV (CMV-LE) (15) had been prepared in mouse ascites fluid (6). Antisera previously prepared in rabbits against PSV-J (16) and PSV-W (10) were supplied by Tsuchizaki and Mink, respectively.

For this study, we prepared antibodies against PSV-J, PSV-K61 (Mink et al., *unpublished strain*), and CMMV (13) in mouse ascites fluid using the method of Kiriyama and Osumi (6). Viruses were partially purified from tobacco leaf tissue by Scott's procedure followed by rate sucrose density-gradient ultracentrifugation. The infectious, visible zones were removed and concentrated into 0.01 M neutral phosphate buffer by one cycle of differential ultracentrifugation. Individual mice were injected interperitoneally three times at weekly intervals with 0.1 ml of PSV-J (0.8 mg/ml) PSV-K61 (0.5 mg/ml) or CMMV (2 mg/ml) emulsified in an equal volume of Freund's adjuvant.

Antigen preparation.—Viruses used as test antigens included three CMV strains; CMV-O (2), CMV-Az (5) and CMV-Lg (4); PSV isolates J and K61; and one CMMV isolate. Each virus was increased in tobacco and partially purified by Scott's procedure. Antigens used for ring interface tests were further purified by rate sucrose density-gradient ultracentrifugation. Visible virus zones were removed and concentrated by one cycle of differential ultracentrifugation. Pellets were suspended in 0.01 M neutral phosphate buffer and filtered through a membrane filter having 30-nm pore size. For ring interface titer tests each antigen was diluted in phosphate buffer to a final concentration of 100 μg/ml as estimated by optical density values. No reactions were obtained with purified virus antigens at concentrations below 200 μg/ml when tested against an antiserum prepared to healthy plant components. The antiserum (titer 1/128) to healthy plant components was diluted 1/40 before use.

Serological tests.—In ring interface tests, plant components prepared from healthy tobacco tissue produced nonspecific reaction lines with all mouse ascites fluids diluted up to 1/64 (Table 1). Virus antigens prepared by Scott's procedure, and containing some residual plant components, also produced this nonspecific reaction with ascites fluids diluted up to 1/16 or 1/32. Virus antigens further purified by density-gradient centrifugation usually did not produce this

TABLE 2. Reciprocal titer of antiserum CMV-YFA before and after absorbing with various antigens

Absorbing antigen	Test antigen		
	CMMV	PSV	CMV
Buffer	16	64	1,024
CMMV[a]	0	64	1,024
PSV[b]	16	0	1,024
CMV[c]	16	64	16

[a]CMMV = chrysanthemum mild mottle virus.
[b]PSV = peanut stunt virus.
[c]CMV = cucumber mosaic virus.

nonspecific reaction with control fluids. Some ascites fluids also produced another type of nonspecific reaction presumably due to the formation of calcium phosphate at the interface. This reaction did not occur when ascites fluids were dialyzed overnight against 0.01 M neutral phosphate buffer, and the resulting precipitate removed by centrifugation.

Gel-diffusion tests were made using a modified microslide technique (8). Agar (0.5%) in neutral saline was allowed to flow at a thickness of one layer of electrical tape between a slide and a plastic template containing predrilled tapered holes in the appropriate design. After storage overnight at 4 C, solidified agar was removed from the holes and the test solutions added. In some tests antigen preparations were used prior to density-gradient centrifugation. For these the antigen content was estimated serologically to be between 0.5 and 1.0 mg/ml for each virus. Antigens used after density-gradient centrifugation contained 0.5, 0.5, 0.8 mg/ml CMV-Az, PSV-J and CMMV, respectively.

RESULTS.—*Direct experiments.*—All strains of CMV used in these tests appeared serologically similar, if not identical, when reacted against any CMV antiserum in gel-diffusion tests. Similar results were obtained when both PSV isolates were tested against PSV antisera or ascites fluid.

Ascites fluids prepared against CMMV, PSV-J, PSV-K61, and CMV-LE (as well as rabbit antisera prepared against PSV) reacted only with antigens of the homologous type in both ring interface (Table 1) and gel-diffusion tests. Using these combinations of reactants we found no indication that any two of the three viruses were related serologically. These results confirm and expand earlier reports on the lack of serological relationship between CMV and CMMV (13), and between CMV and PSV (9). However, these results differ somewhat from an earlier report (9) where PSV-W was found to be related to an American aspermy-type virus from chrysanthemum. This lack of relationship among viruses was supported by cross-protection tests. None of the three viruses completely protected cowpea plants from infection by the other two viruses (Iizuka, *unpublished*).

Even though CMV, PSV and CMMV appear to be unrelated serologically, we found that five antisera prepared against two CMV strains reacted not only with purified CMV, but also with PSV and CMMV (Table 1). Three of these antisera were prepared against the same isolate of CMV-O, and contained reciprocal titers of CMV-specific antibodies between 2,048 and 4,096. Each CMV-O antiserum reacted with PSV and CMMV preparations at dilutions up to 1/16 in ring interface tests. As expected, these antisera produced strong reaction lines in gel-diffusion tests with all CMV isolates, but produced only faint or no reactions with either PSV or CMMV.

Two antisera prepared against an isolate of CMV-Y (14) contained high titers of CMV antibodies, and moderately high titers of PSV antibodies (Table 1). The titer of CMMV antibodies was low in both antisera. In gel-diffusion tests both CMV-Y antisera produced strong reaction lines with CMV and with PSV. In most tests, both the slow-moving, presumably high molecular weight antigen and the fast-moving, low molecular weight antigen previously described for CMV (12) and PSV (9) were observed. Purified CMMV reacted weakly with either CMV-Y antiserum, but two antigen species could usually be observed. Despite the fact that all three viruses reacted with CMV-Y antisera, in every case where precipitin lines developed clearly enough to determine relationships, we observed only distinct crossing of the lines indicating that no two of the three viruses possessed common antigens.

TABLE 3. Reciprocal end point of cucumber mosaic virus (CMV) and peanut stunt virus (PSV) antigens in a partially purified preparation of CMV-Lg before and after absorbing with various antisera

Absorbing antiserum	Test antiserum[a]	
	CMV	PSV
None	160	80
Healthy	160	80
CMV-LE[b]	0	80
CMV-YFA (Absorbed with PSV)	0	80
CMV-OA	0	40

[a]CMV antiserum = CMV-YFA diluted 1/500, PSV antiserum = PSV-W diluted 1/40.
[b]Ascites fluid.

Absorption experiments.—The above data suggest that the antibodies for each of the three viruses present in the CMV-Y antisera are not connected physically. We tested this possibility by absorbing CMV-YFA antiserum with single viruses and determining the titer of the remaining antibodies. Undiluted CMV-YFA antiserum (0.4 ml) was mixed with 0.2 ml of the following: (i) phosphate buffer, (ii) purified CMV-LE (0.2 mg/ml), (iii) purified PSV-J (0.3 mg/ml), or (iv) purified CMMV (0.4 mg/ml). After incubation at 20 C for 48 hours, each solution was diluted with 0.2 ml phosphate buffer (final antiserum dilution = 1/4), filtered through a membrane filter, diluted serially in phosphate buffer-10% glycerine-saline and tested in ring interface tests. Although the CMV concentration used was too low to remove all CMV antibodies, the results (Table 2) indicate that only antibodies against a single virus were removed in each absorbing combination. This strongly supports the hypothesis that three separate antibody species are present in the CMV-Y antisera.

Some possible reasons for the occurrence of a mixture of antibodies in some CMV antisera were discussed earlier (9). The most obvious explanation is that a mixture of viruses was used for injection. Because the antisera we used here had been prepared 1-2 years before these tests were made, we were unable to examine the original antigen solutions for their virus content. However, we did test two isolates of CMV-Y that were derived from the same original source as the isolate used to prepare the CMV-Y antisera. We detected no PSV or CMMV antigens by either ring interface or gel-diffusion tests in partially purified preparations of one isolate that contained approximately 1.0 mg/ml virus. The second purified CMV-Y isolate reacted with PSV antisera when tested undiluted (about 1.0 mg/ml) in gel-diffusion tests. This isolate also reacted with PSV antisera in ring interface tests when diluted to 100 μg/ml. This same CMV-Y isolate reacted with CMMV ascites fluid when

tested undiluted in gel-diffusion tests, but did not react when diluted to a final concentration of 100 μg/ml for ring interface tests.

We consistently detected PSV serologically in both crude sap and partially purified preparations from cowpea tissue infected with CMV-Lg strain. Occasionally CMMV was also detected, especially in partially purified preparations. We used this CMV isolate to determine if absorption of CMV by various CMV antisera resulted in a decrease in PSV antigen titer. A partially purified preparation which initially reacted with both CMV and PSV antisera was absorbed with (i) ascites fluid CMV-LE which contained no PSV antibodies, (ii) antiserum CMV-FYA from which the PSV antibodies had been previously absorbed, and (iii) antiserum CMV-OA which contained a low amount of PSV antibodies in addition to CMV antibodies (see Table 1). The results (Table 3) indicate that when CMV antibodies only were used, all CMV antigens were removed from the mixture without decreasing the titer of PSV antigens. This supports the hypothesis that both CMV and PSV were present in the purified preparation of CMV-Lg.

DISCUSSION.—The earlier demonstration (9) that PSV-specific antibodies occur in a number of CMV antisera from many sources, together with the results of this study, suggests that antisera to mixtures of CMV and other viruses may be produced more often than was previously suspected. So far as we are aware, this is the first demonstration that antibodies specific for an aspermy-type virus also occur in some CMV antisera.

Even though our data suggest that virus mixtures are a logical explanation for the fact that some CMV antisera contain mixtures of antibodies, we have no evidence as to how such mixtures arise. Each virus studied here is readily transmitted by aphids, and in some plants they may cause similar symptoms. Consequently it would not be surprising to find natural mixtures of these viruses.

Some isolates of CMV-Y and PSV can be differentiated by the lesions they produce on cowpea leaves. Yet, prior to this study and during it we saw no symptomatological evidence that the CMV-Y isolate was contaminated with PSV. The reason for this is not known.

The results of these studies raise some question as to the validity of earlier studies on serological relationships among viruses in the CMV group. The so-called "broad spectrum" and "narrow spectrum" antisera used to substantiate claims of relationship between CMV and the aspermy-type viruses have been identified by their ability to react with heterologous viruses. Described with these imprecise terms, our ascites fluid against CMV-LE would be considered "narrow spectrum", whereas the antisera against CMV-O and CMV-Y, particularly the latter, would be considered "broad spectrum". However, such usage obscures the fact that antibodies specific for three unrelated viruses coexist in the same antiserum. Consequently, apparent relationships among the viruses will vary according to the number of antigen species present in the isolates tested. It seems obvious that results from gel-diffusion tests alone which show serological relationships among viruses in the CMV-TAV-PSV group should be interpreted with caution.

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