Serological Differentiation of Some Strains of Alfalfa Mosaic Virus with Polyclonal Antibodies

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ABSTRACT

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Five biologically distinct but antigenically similar alfalfa mosaic virus (AMV) strains were tested for minor antigenic differences by immunodiffusion tests and indirect enzyme-linked immunosorbent assay (ELISA) by using antisera elicited to native and glutaraldehyde-fixed AMV particles of each of the virus strains. In addition, antisera to coat protein preparations of two of the strains were also used. In immunodiffusion tests with antisera to fixed virus particles and either fixed or native virus preparations as test antigens, no antigenic differences were detected among any of the strains. However, antisera to native AMV particles revealed antigenic differences among all five strains, although some were easier to detect than others. The differences were more readily revealed, however, when fixed virus preparations were used as test antigens. Similar differences were also detected when antisera elicited to AMV coat protein preparations were used. In general, strain-specific differences among the AMV strains were more difficult to detect by indirect-ELISA. It is concluded that some antibodies to strain-specific epitopes on AMV are present in antisera elicited in response to native AMV particles or their isolated coat proteins, but that these epitopes are more easily detected when fixed particles are used as test antigens. It is also concluded that the strain-specific epitopes were metatopes.

Numerous isolates of alfalfa mosaic virus (AMV) can be distinguished by symptomatology (5,15,18,19), particle lengths distribution (19,31), cytopathic differences (7,20), coat protein amino acid composition (22), coat protein mRNA leader sequences (36), 5′-end structures of their genomic RNAs (23,28), and their in vitro translation products (10). In spite of such differences, the variants have been shown to be remarkably uniform in their antigenic properties (2,11,15,18,26,27,37,44). There are two reports in which AMV variants were distinguished in immunodiffusion tests, but in these instances the antiserum had been raised against purified AMV particles that had been heated for 1 h at 30 C before injection into the rabbits (29,42).

More recently, we have reported that some AMV strains can be distinguished with monoclonal antibodies (14). In this paper we show that the AMV strains can also be distinguished with antisera elicited in rabbits by using preparations of either native AMV particles or their isolated coat proteins.

MATERIALS AND METHODS

Virus strains and their purification. The H4, N20, S30, S40, and W1 strains of AMV isolated from lucerne in South Australia were propagated in *Nicotiana clevelandii* Gray. They were all readily distinguishable by their biological properties (15). The viruses were purified by differential and sucrose density-gradient centrifugation and, when required, were fixed by the addition of glutaraldehyde to 0.25% (v/v) (15).

Preparation of AMV coat protein and host leaf antigen. Highly purified virus was dissociated with $CaCl_2$, and *N. clevelandii* leaf extract was prepared as previously described (14,16).

Antisera. All antisera were prepared in rabbits. Those to glutaraldehyde-fixed AMV strains were the same as used previously (15). Rabbits were also immunized by the same schedule of injections with preparations of the five AMV strains without fixation (antisera to native virus strains). Only antisera from two selected bleedings from each of the 10 rabbits were used, referred to as "early" and "late." Each "early" antiserum was that from the earliest bleeding in which the titer reached a maximum (1/16 or greater) when tested by immunodiffusion against 200 μg/ml of its homologous glutaraldehyde-fixed AMV strain. This varied from 8 to 17 wk after the initial immunization (five to six injections). "Late" antisera were those collected 22–23 wk after initial immunization (seven injections) that had titers between 1/16 and 1/256.

Antisera to viral coat protein preparations of the S30 and S40 AMV strains were the same as those used before (16). Only one antiserum from each of the four rabbits (two immunized with protein of the S30 and two of the S40 AMV) was used, collected 10 or 11 wk after the first injection. Titers of these antisera are presented in the Results (see Table 2 below).

Gel-immunodiffusion tests. Tests with native or glutaraldehyde-fixed AMV preparations as antigens were done in 0.75% agar or agarose buffered with 10 mM sodium phosphate, pH 7.6, containing 0.02% (w/v) sodium azide (15). In experiments in which AMV coat protein preparations were used as test antigens, 0.75% agarose gels in 10 mM sodium acetate, pH 7.6, containing 100 mM $CaCl_2$ and 0.02% (w/v) sodium azide were used (16). Native or glutaraldehyde-fixed virus preparations were diluted in 10 mM phosphate buffer, pH 7.0, and coat protein preparations in 10 mM sodium acetate, pH 6.0, containing 100 mM $CaCl_2$. Antisera were diluted in the same buffer as that used for preparation of the gels. Unless otherwise stated, virus or coat protein concentrations of 200 μg/ml were used, and each 3-mm-diameter well in the gel was charged with 10 μl of antigen or antiserum. All reactions were recorded after 5 days of incubation at 25 C.

The serological differentiation index (SDI) of two virus strains is here defined as the number of twofold dilution steps separating homologous from heterologous titers in immunodiffusion tests (40).

Intragel cross-absorption tests. The central wells of the gel plates were charged with 10 μl of a 1 mg/ml preparation of the cross-absorbing antigen and the plates were incubated at 25 C for 16 h.

The same well was then filled with 15 μl of antiserum and the outer wells were charged with 15 μl of the antigen to be tested, adjusted to a concentration of 200 μg/ml. The plates were incubated for a further 5 days before recording the results.

Indirect enzyme-linked immunosorbent assay (ELISA). Aliquots of 200 μl of glutaraldehyde-fixed or native AMV preparations diluted to 0.5 μg/ml in carbonate coating buffer, pH 9.6 (4), were applied to microtiter wells and incubated for 3 h at 25 C. Under these conditions both fixed and native AMV have a high affinity for polystyrene (13). Viral coat protein preparations were diluted to concentrations of 20 ng/ml in 10 mM sodium acetate buffer, pH 6.0, containing 100 mM $CaCl_2$ and incubated at 25 C for 3 h (16). The wells were then rinsed and subsequently blocked with 350 μl of blocking solution (0.1 M NaCl containing 1% [w/v] bovine serum albumin [BSA]) and incubated for 1 h at 25 C. The plates were again rinsed, and twofold dilutions (200 μl) of antisera in PBS-Tween, pH 6.0 (4), containing 2% (w/v) polyvinylpyrrolidone (PVP) and 0.02% (w/v) sodium azide were added to each well. After 16 h at 4 C the wells were again rinsed and charged with 200 μl of a 1:1,000 dilution of affinity purified goat anti-rabbit IgG labeled with alkaline phosphatase in conjugate buffer (PBS-Tween, pH 7.4, containing 2% [w/v] PVP, 0.2% [w/v] BSA and 0.02% [w/v] sodium azide). After 3 h at 25 C, the wells were again rinsed and charged with 200 μl of 1 mg/ml of *p*-nitrophenyl phosphate in diethanolamine substrate solution, pH 9.8, and the optical density at 405 nm was determined with a Bio-Rad Model 2550 EIA reader (Bio-Rad, Richmond, CA). Rinsing of the wells mentioned above were each done three times, each for 3 min with 350 μl of PBS-Tween, pH 7.4. The parameters of the indirect ELISA were optimized as described by Jaegle and Van Regenmortel (21).

RESULTS

Immunodiffusion tests. The titers of all the antisera used in immunodiffusion tests were generally higher when reacted with glutaraldehyde-fixed AMV than with native virus preparations, irrespective of whether the antisera had been raised against fixed or native virus (Table 1).

Tests with the antisera to fixed AMV (Table 1) show that a few of the heterologous titers differed from the homologous by an SDI rating of 1. This is not considered as significant. The only heterologous titer that exceeded this was one of the antisera to AMV-H4 that, when tested with native virus preparations of the S30, S40, and W1 strains of AMV, produced an SDI rating of 2 (Table 1). This suggests a possible antigenic difference between H4-AMV and the S30, S40, and W1 strains of the virus.

The antisera to fixed virus particles were also subjected to immunodiffusion tests with each antiserum placed in a central well surrounded by wells containing virus preparations as shown in Figure 1. The antiserum to fixed AMV (titer 1/64) was diluted 1/8 and that of the native virus (1/32) was diluted 1/4, and the virus preparations were adjusted to 500 μg/ml. The assays were done so that each antiserum was tested against homologous and

TABLE 1. Reactions among antisera to glutaraldehyde-fixed and native alfalfa mosaic virus (AMV) strains with their homologous and heterologous antigens in immunodiffusion tests[a]

Antiserum elicited to[b]	Test antigen[c]									
	Fixed AMV strain					Native AMV strain				
	H4	N20	S30	S40	W1	H4	N20	S30	S40	W1
Fixed AMV - H4										
Early	*64*[d]	64	64	64	64	*16*	8(1)	4(2)	4(2)	4(2)
Late	*64*	64	64	64	64	*16*	8(1)	8(1)	16	8(1)
Fixed AMV - N20										
Early	32	*32*	32	32	32	4	*4*	4	4	4
Late	64	*64*	32(1)	64	32(1)	8	*8*	8	8	8
Fixed AMV - S30										
Early	64	64	*64*	64	64	8	8	*8*	8	8
Late	128	128	*128*	128	128	16	16	*16*	16	16
Fixed AMV - S40										
Early	128	128	128	*128*	128	16	16	16	*16*	16
Late	256	256	256	*256*	256	32	32	32	*32*	32
Fixed AMV - W1										
Early	64	64	64	32(1)	*64*	8	8	8	8	*8*
Late	64	64	64	64	*64*	8	8	8	8	*8*
Native AMV - H4										
Early	*32*	32	4(3)*	8(2)*	4(3)*	*4*	4	4	4	4
Late	*32*	32	4(3)*	8(2)*	16(1)*	*16*	16	16	16	16
Native AMV - N20										
Early	16	*16*	8(1)	4(2)*	4(2)*	16	*16*	16	8(1)	8(1)
Late	16	*16*	16	4(2)*	4(2)*	16	*16*	16	8(1)	8(1)
Native AMV - S30										
Early	4(3)*	8(2)*	*32*	8(2)*	8(2)*	4(2)*	8(1)*	*16*	4(2)*	8(1)*
Late	8(2)*	8(2)*	*32*	8(2)*	4(3)*	4*	4*	*4*	4*	4
Native AMV - S40										
Early	16	16	16	*16*	8(1)	4	2(1)	2(1)	*4*	4
Late	32	32	32	*32*	32	8	8	4(1)	*8*	4(1)
Native AMV - W1										
Early	4(4)*	64	32(1)	64	*64*	16(1)	16(1)	16(1)	8(2)	*32*
Late	8(3)*	64	64	64	*64*	4(1)	4(1)	4(1)	4(1)	*8*

[a] Immunodiffusion tests were done in 0.75% agarose gels buffered with 10 mM sodium phosphate buffer, pH 7.6, containing 0.02% (w/v) sodium azide.

[b] Antisera prepared as described in Materials and Methods.

[c] Virus preparations were adjusted to 200 μg/ml and 10 μl was dispensed into each well as described in Materials and Methods.

[d] Numbers indicate reciprocals of maximum dilutions of antisera at which immunoprecipitin lines were detected (italics indicating homologous reactions). Numbers in parentheses indicate serological differentiation index ratings (SDI = number of twofold dilution steps separating homologous and heterologus titers). Asterisks indicate that spurs were formed when antisera were tested against preparations of homologous and heterologous virus strains in adjacent wells of immunodiffusion plates (see Fig. 1B).

heterologous virus strain preparations placed in adjacent wells. These tests with several different virus preparations consistently failed to detect the formation of spurs between any pair of virus strains, whether they were fixed or not (Fig. 1A and data not shown). Thus, no serological differences between any of the AMV strains were detected in these experiments when antisera to fixed AMV were used.

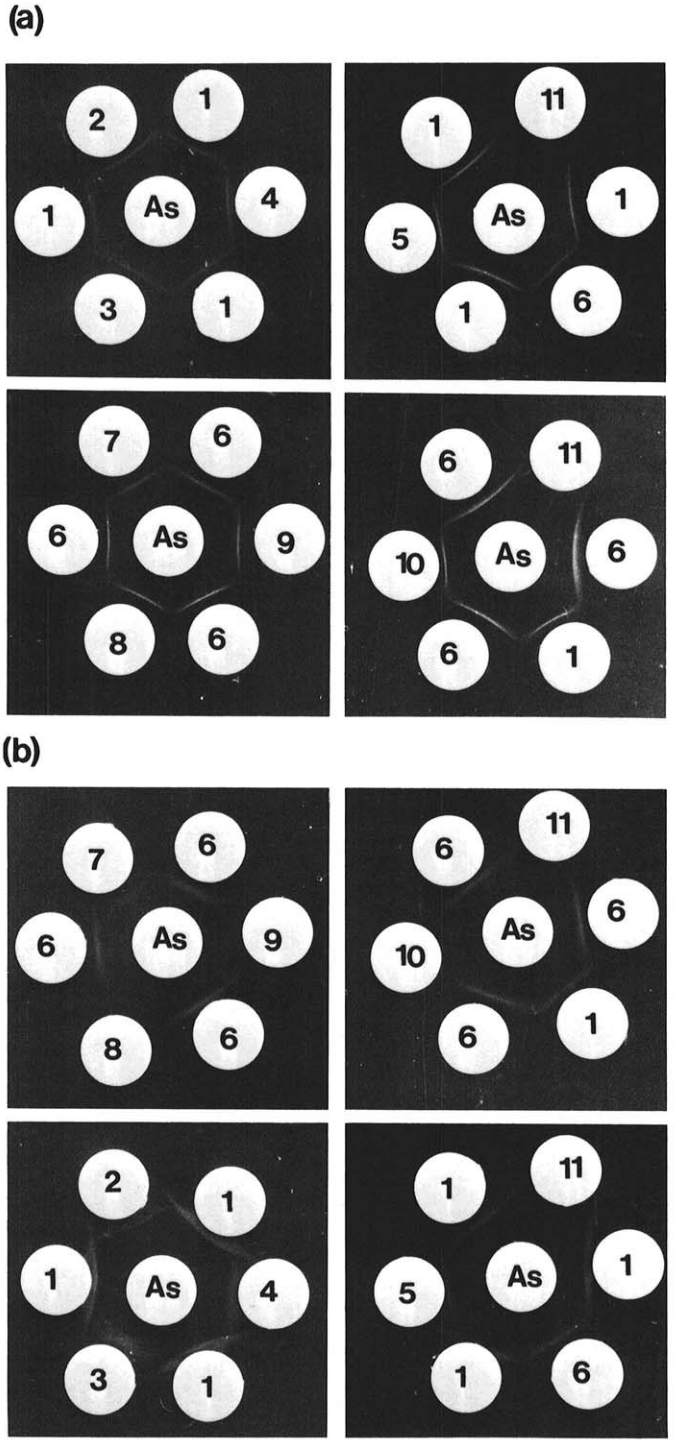

Fig. 1. Immunodiffusion tests in agarose gels among antisera from early bleedings (As) to **A,** glutaraldehyde-fixed and, **B,** native AMV-S30 reacting with fixed (1–5) and native virus preparations (6–10) of strains S30, S40, N20, H4 and W1, respectively. Well 11 was filled with a concentrated protein preparation from healthy *Nicotiana clevelandii* leaves. Antiserum to fixed AMV-S30 (titer 1/64) and to native AMV-S30 (titer 1/32) were diluted 1/8 and 1/4, respectively, in 10 mM sodium phosphate buffer, pH 7.0. Virus preparations were adjusted to 500 μg/ml, and the wells were charged with 10 μl of each reactant.

The results of similar experiments with antisera to native virus particles reacting with fixed virus preparations show that 19 of the heterologous titers differed by an SDI rating of 2 to 4 (Table 1). These data suggested that there are antigenic differences among all the AMV strains, except between the H4 and N20 and the S40 and W1 strains (Table 1). These differences were confirmed by the formation of spurs in tests such as those shown in Figure 1B and summarized in Table 1. It is noteworthy that far fewer of the antigenic differences among the AMV strains were detected when the same antisera were tested against native preparations of the virus strains. Nevertheless, antisera to S30 AMV differentiated this virus from the remaining four strains (Fig. 1B and Table 1).

Antisera elicited in response to coat protein preparations from the S30 and S40 AMV strains were also used in immunodiffusion tests that used fixed and native virus preparations of the five virus strains as well as their coat proteins as test antigens. The results summarized in Table 2 show that although very few of the heterologous titers exceeded an SDI rating of 1, some of the virus strains were differentiated from S30 and S40 AMV by detection of spurs between heterologous virus strains. These strain differences were more readily detected with antisera from some rabbits than others when native (Fig. 2) or fixed (Table 2) virus preparations were used as test antigens. Coat protein preparations appeared to be the least useful for the detection of strain differences in these tests, and only the H4 and S40 AMV strains were differentiated by one of the two antisera raised against coat protein of AMV-S40 (Table 2).

Intragel cross-absorption tests. The results of the immunodiffusion experiments described above showed that serological differences among AMV strains were most readily revealed when tests were done with antisera to native virus strains and fixed virus preparations as test antigens (Table 1). Thus, we used the same reagents in intragel cross-absorption tests. The results summarized in Table 3 show that, although many of the antisera were exhausted of antibodies when cross-absorbed with some heterologous antigens, others were not. Moreover, in some instances, precipitin lines were formed with spurs when an antiserum cross-absorbed with one heterologous virus strain was reacted against preparations of the homologous and another heterologous virus strain placed in adjacent wells. Data summarized in Table 3 show that all the virus strains could be differentiated from each other, except strain S40 from W1.

Antisera from two rabbits immunized with coat protein preparations of the S30 and two with the S40 AMV strains were also used in similar cross-absorption experiments with fixed and native virus preparations as test antigens. It is noteworthy that both antisera to S40-AMV when tested against a preparation of fixed W1-AMV still produced a precipitin line revealing an antigenic difference between these two AMV strains. Results in Table 4 show that all the AMV strains were distinguished from each other when fixed virus preparations were used as test antigens. However, with native virus preparations, the S40 strain of AMV was not differentiated from W1 AMV (Table 4).

Data in Tables 2 and 4 also show that antisera to the same viral antigen but from different animals varied in their ability to detect antigenic differences between different AMV strains.

Indirect ELISA. Fixed and native AMV as well as coat protein preparations of the five virus strains were used to coat ELISA microtiter plates. These were then reacted with twofold dilution series of antisera elicited to both fixed and native preparations of all the five virus strains, and to the coat proteins of the S30 and S40 strains. Only the results of experiments with the three different antisera to S30 AMV are presented in detail (Fig. 3); results of all the tests are summarized in Table 5.

Results of these experiments show that the antisera to fixed virus particles failed to detect any antigenic differences among any of the five virus strains when fixed virus preparations were used as test antigens (Fig. 3A, Table 5). However, some differences became evident when either native virus or coat protein preparations were used (Fig. 3B and C, Table 5). The antiserum to S30 AMV was especially useful as it distinguished S30 AMV

TABLE 2. Reactions among antisera to coat protein preparations of alfalfa mosaic virus (AMV) strains with homologous and heterologous virus and coat protein antigens in immunodiffusion tests[a]

Antiserum elicited to[b]	Test antigen[c]														
	Fixed AMV strain					Native AMV strain					Coat protein of AMV strain				
	H4	N20	S30	S40	W1	H4	N20	S30	S40	W1	H4	N20	S30	S40	W1
S30-AMV (Rabbit a)	16(1)[d] *	16(1)*	*32*	16(1)*	16(1)*	8(1)*	8(1)*	*16*	8(1)*	4(2)*	2(1)	2(1)	*4*	4	2(1)
S30-AMV (Rabbit b)	4(1)*	4(1)*	*8*	4(1)*	4(1)*	4	4*	*4*	1(2)*	1(2)*	1	1	*1*	1	1
S40-AMV (Rabbit c)	64(1)*	64(1)*	64(1)	*128*	128	32(1)*	32(1)*	32(1)*	*64*	64	4(1)*	4(1)	8	*8*	8
S40-AMV (Rabbit d)	64	32(1)	64	*64*	32(1)	8*	8*	8*	*8*	8	8	4(1)	8	*8*	4(1)

[a] Tests done as described under Materials and Methods and Table 1.
[b] Antisera from late bleedings (11–12 wk after initial immunization) were used (15).
[c] All antigen preparations were adjusted to 250 μg/ml and used as described under Materials and Methods and Table 1.
[d] Numbers indicate reciprocals of maximum dilutions of antisera at which immunoprecipitin lines were detected (italics indicating homologous reactions). Numbers in parentheses indicate serological differentiation index ratings (SDI). Asterisks indicate that spurs were formed when antisera were tested against preparations of homologous and heterologous virus strains in adjacent wells of immunodiffusion plates (see Fig. 2).

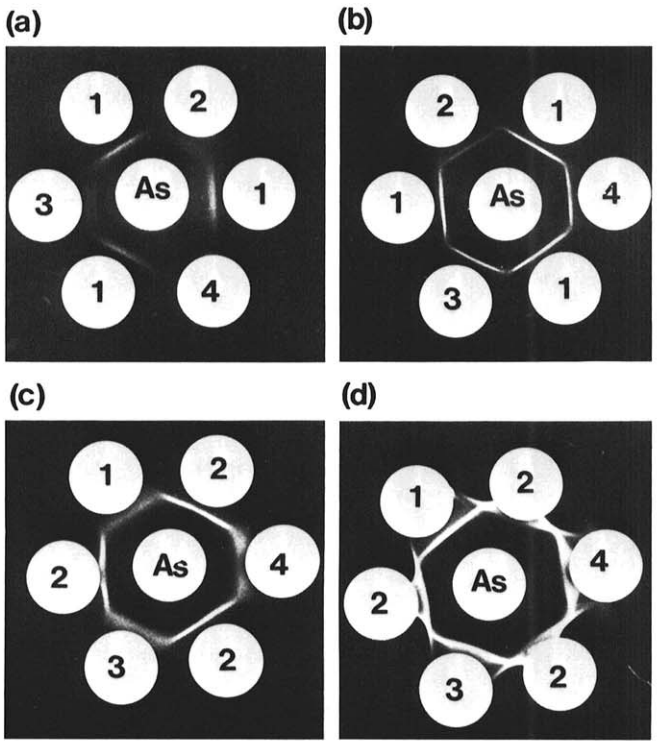

Fig. 2. Immunodiffusion tests in agarose gels with antisera (As) from four rabbits immunized with coat protein preparations of **A and B,** AMV-S30, **C and D,** AMV-S40. Central wells (As) were charged with each antiserum and peripheral wells (1–4) with a preparation of native alfalfa mosaic virus strains, S30, S40, H4, and N20 adjusted to 250 μg/ml, respectively. Titers of the antisera used are presented in Table 2. The wells were charged with 10 μl of each reactant.

TABLE 3. Analysis of alfalfa mosaic virus (AMV) strains by intragel cross-absorption tests with antisera to native virus particles[a]

Antiserum elicited to AMV strain[b]	Antiserum cross-absorbed with AMV strain	Test antigen (fixed AMV strain)[c]				
		H4	N20	S30	S40	W1
H4	H4 (control)	−[c]	−	−	−	−
	N20	−	−	−	−	−
	S30	+	+	−	−	−
	S40	+	+	−	−	−
	W1	+	+	−	−	−
N20	N20 (control)	−	−	−	−	−
	H4	−	−	−	−	−
	S30	−	−	−	−	−
	S40	+	+	+	−	−
	W1	+	+	+	−	−
S30	S30 (control)	−	−	−	−	−
	H4	−	−	+	++	++
	N20	−	−	+	++	++
	S40	++	++	+	−	−
	W1	++	++	+	−	−
S40	S40 (control)	−	−	−	−	−
	H4	−	−	−	−	−
	N20	−	−	−	−	−
	S30	−	−	−	−	−
	W1	−	−	−	−	−
W1	W1 (control)	−	−	−	−	−
	H4	−	+	+	+	+
	N20	−	−	−	−	−
	S30	−	−	−	−	−
	S40	−	−	−	−	−

[a] The central well of each gel plate was charged with 10 μl of a 1 mg/ml of the appropriate fixed virus preparation and the plates were incubated at 25 for 16 h. The same wells were then charged with 15 μl of antiserum and the outer wells were charged with 15 μl of the test antigen adjusted to a concentration of 200 μg/ml.
[b] Antisera from early bleedings were used (see Table 1 for titers).
[c] Absence of visible precipitin lines is indicated by −, presence of a line by +, and ++ indicates that precipitin lines were formed with spurs when an antiserum cross-absorbed with one heterologous virus was reacted against preparations of the homologous and another heterologous strain placed in adjacent wells.

as antigenically distinct from all the other four virus strains when either their native virus or coat protein preparations were used.

All the antisera to native virus particles failed to reveal any antigenic differences among any of the AMV strains, whether or not fixed or native virus preparations were used as antigens (Fig. 3D and E, Table 5). However, a few of the strains were distinguished from each other when these antisera were tested with preparations of the coat proteins, and again the antiserum to S30 AMV was the most discriminatory (Fig. 3F, Table 5).

One of the two antisera to the coat protein of S30 AMV (from rabbit b) was able to distinguish S30 AMV from the S40 and W1 strains of AMV, but only when coat protein preparations were used as coating antigen (Table 5). However, the other antiserum to this antigen (rabbit a) failed to distinguish S30-AMV from any of the other virus strains. Thus, the two antisera to the same immunogen, coat protein of S30-AMV, differed in their ability to distinguish antigenic differences among the AMV strains. The two antisera to coat protein of S40 AMV were completely

nondiscriminating with preparations of either fixed or native virus or with coat proteins used for coating the microtiter plates (Table 5).

From all the results summarized in Table 5, the existence of minor antigenic differences was revealed among all five virus strains. These were most readily detected with antisera to the fixed virus strains where coat protein preparations, and to a lesser extent native virus particles as test antigens, were used. Some differences were also detected with antisera to native AMV and coat protein as test antigens.

Preparations of some strain-specific and group-specific antisera. Data presented in Tables 3–5 revealed that in addition to many common epitopes, the five AMV strains also had epitopes that were either unique or shared among some, but not all, of the other strains. This prompted us to investigate the possibility of preparing strain-specific antisera to some of the viruses by cross-absorption with heterologous virus preparations in liquid medium. For example, we were successful in preparing an antiserum that reacted with S30 AMV but not with any of the other four virus strains when fixed virus preparations were compared. This was done by adding 150 μg each of the four heterologous virus strains that had been fixed with glutaraldehyde to each milliliter of antiserum elicited to native particles of S30-AMV. After incubation and removal of the virus-antibody precipitate by centrifugation, the antibodies remaining in the supernatant reacted only with the S30 strain of AMV (Fig. 4A). Similarly, cross-absorption of an antiserum to native H4 AMV particles with a mixture of preparations of the S30, S40, and W1 virus strains that had been fixed resulted in an antiserum that reacted only with the N20 and H4 strains of AMV (Fig. 4B). In yet another experiment, an antiserum to native W1 AMV particles, when absorbed with a preparation of fixed H4 AMV, yielded a group-specific antiserum that reacted with the W1, N20, S30, and S40 but not with the H4 strain of AMV (Fig. 4C). However, the titers of these cross-absorbed antisera were low and hence of limited use.

DISCUSSION

In spite of the close antigenic relationships among all the five AMV strains studied, they were all distinguished from each other with selected antisera by at least one type of immunodiffusion test. The differences among some strains were much easier to demonstrate than among others. For example, S30 AMV was relatively easily distinguished from the other four strains of the virus so that a S30 AMV-specific antiserum could be prepared by cross-absorption. On the other hand, the antigenic difference between the S40 and W1 strains of AMV could only be revealed by one of the tests in which antisera to the S40 AMV coat protein was cross-absorbed with a preparation of fixed W1 AMV (Table 4).

Data presented in this paper show that in immunodiffusion tests, antisera elicited to fixed AMV particles revealed no antigenic differences among any of the different virus strains, irrespective of whether fixed or native virus preparations were used as test antigens (Table 1). On the other hand, antisera to native AMV particles revealed antigenic differences among some of the AMV strains, but the differences were much more clearly seen when fixed virus preparations were used as test antigens (Table 1). Similar differences were also revealed when antisera elicited to AMV coat proteins were used (Table 2). These observations indicate that in immunodiffusion tests, more antibodies to strain-specific epitopes are present in antisera elicited in response to native AMV particles or their isolated coat protein but that these specific epitopes are more easily revealed when fixed virus particles are used as test antigens.

It has been demonstrated that glutaraldehyde fixation stabilizes the structure of AMV particles without introducing significant antigenic changes (13) and, hence, such particles expose only their metatopes and neotopes. Thus, it seems most unlikely that strain-specificity is determined by cryptotopes because these are not exposed during immunodiffusion. It is also unlikely that the neo-

TABLE 4. Analysis of alfalfa mosaic virus (AMV) strains by intragel cross-absorption tests with antisera to viral coat proteins[a]

Antiserum elicited to protein of[b]	Antiserum cross-absorbed with AMV	Test antigen: Fixed AMV strain H4	N20	S30	S40	W1	Native AMV strain H4	N20	S30	S40	W1
S30-AMV (Rabbit a)	S30 (control)	−[c]	−	−	−	−	−	−	−	−	−
	H4	−	−	+	−	−	−	−	+	−	−
	N20	−	−	+	++	−	−	−	+	−	−
	S40	−	−	+	−	−	−	−	+	−	−
	W1	−	−	+	−	−	−	−	+	−	−
S30-AMV (Rabbit b)	S30 (control)	−	−	−	−	−	−	−	−	−	−
	H4	−	++	+	−	−	−	−	+	−	−
	N20	−	−	−	−	−	−	−	+	−	−
	S40	−	++	+	−	−	−	−	+	−	−
	W1	−	++	+	−	−	−	−	+	−	−
S40-AMV (Rabbit c)	S40 (control)	−	−	−	−	−	−	−	−	−	−
	H4	−	−	+	+	−	−	−	−	+	+
	N20	++	−	++	+	−	−	−	−	+	+
	S30	++	−	−	+	−	−	−	−	+	+
	W1	++	−	++	+	−	−	−	−	−	−
S40-AMV (Rabbit d)	S40 (control)	−	−	−	−	−	−	−	−	−	−
	H4	−	−	−	+	−	−	−	−	−	−
	N20	+	−	++	+	−	−	−	−	−	−
	S30	++	−	−	+	−	−	−	−	−	−
	W1	+	−	−	+	−	−	−	−	−	−

[a] The central wells were each charged with 10 μl of the appropriate cross-absorbing antigen and the plates were incubated at 25 C for 16 h. The wells were then charged with 15 μl of antiserum and the outer wells were filled with 15 μl of the appropriate test antigen at a concentration of 200 μg/ml.

[b] Antisera from later bleedings (11–12 wk after initial immunization) were used (see Table 2).

[c] Absence of visible precipitin lines is indicated by −, presence of a line by +, and ++ indicates that precipitin lines were formed with spurs when an antiserum cross-absorbed with one heterologous virus was reacted against preparations of the homologous and another heterologous strain placed in adjacent wells.

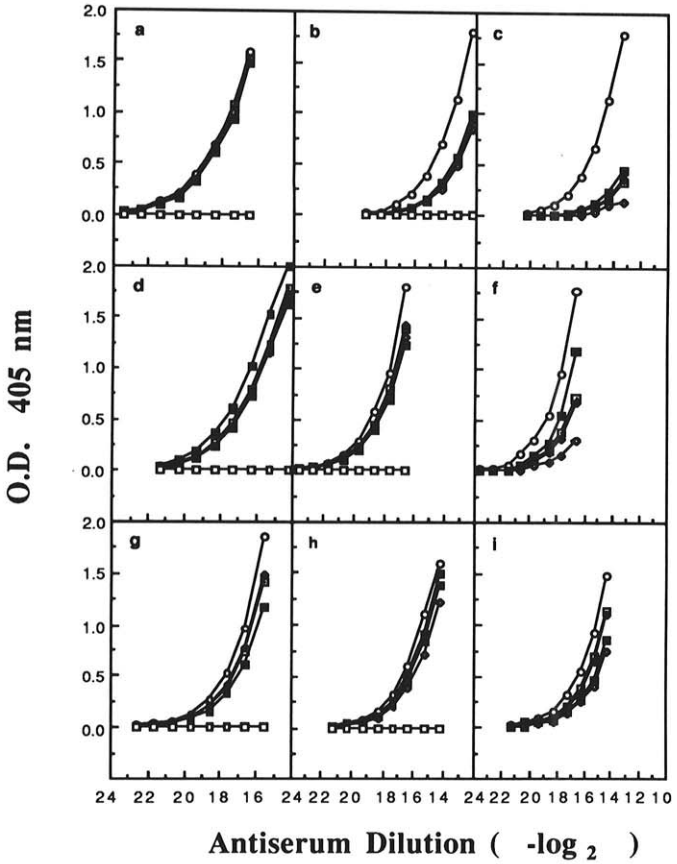

Fig. 3. Serological comparisons of alfalfa mosaic virus (AMV) strains with **A, D, G,** glutaraldehyde-fixed, **B, E, H,** native virus preparations and, **C, F, I,** isolated coat proteins by indirect enzyme-linked immunosorbent assay (ELISA). Antisera prepared against glutaraldehyde-fixed (A–C), native virus preparations (D–F), and isolated coat proteins (G–I) of AMV-S30 were used for detection. AMV strains H4 (⊡), N20 (◆), S30 (○), S40 (◇), and W1 (■) were used as test antigens, and a preparation of tobacco ringspot virus (□) was used as a control antigen. Antisera from early bleedings were used. The microtiter plates were coated with fixed or native virus adjusted to 0.5 μg/ml in carbonate buffer, pH 9.6, and the viral coat protein preparations to 20 ng/ml in 10 mM sodium acetate buffer, pH 6.0, containing 100 mM $CaCl_2$.

topes are involved because antisera raised against fixed particles were not strain-specific. Therefore, it seems that strain differences are principally due to reactions of antibodies recognizing metatopes. Antisera elicited to either native virus particles or coat protein subunits would be expected to contain relatively high concentrations of antibodies reacting with metatopes (13). However, if metatopes are indeed responsible for the strain-specificity, it is puzzling why such antibodies appear to be absent in antisera elicited to fixed virus particles. One possible explanation is that non-strain-specific neotopes on intact AMV particles stabilized by fixation may be immunodominant over any immunogenic strain-specific metatopes. It is also possible that antibodies to strain-specific metatopes are indeed elicited by fixed particles but are unable to react with the particles in immunodiffusion tests because of steric hindrances due to antibodies reacting with the neotopes on the particles. Antisera elicited by fixed virus particles would be expected to be rich in antibodies to neotopes. The observation that some antibodies in antisera to AMV coat protein preparations were capable of recognizing epitopes on fixed but not native AMV preparations (13) adds credence to this suggestion.

In general, strain-specific differences were not as readily revealed among AMV strains in indirect ELISA (Table 5). As in immunodiffusion tests, no antigenic differences were detected by ELISA with antisera to fixed AMV particles when fixed virus preparations were used as test antigens. However, contrary to the immunodiffusion test results, indirect ELISA failed to reveal any antigenic differences among any of the strains with antisera elicited to native AMV particles, irrespective of whether fixed or native virus preparations were used as test antigens.

The apparent discrepancy in revealing antigenic differences among AMV strains by immunodiffusion tests and by indirect ELISA may be at least in part explained by the nature of antibody-antigen reactions in the two different types of tests. During immunodiffusion, reacting native particles probably remain largely intact, whereas when used for coating in ELISA at pH 9.6, they must be completely dissociated into subunits (43). Consequently, the strain-specific epitopes in ELISA may not retain similar conformations to those reacting during immunodiffusion. Furthermore, it is known that adsorption of particles to polystyrene of ELISA wells can lead to changes in protein conformation and hence antigenicity (1,6,12,24,25,34,38). Moreover, during immunodiffusion all antigen surfaces are exposed to antibodies, whereas in ELISA some epitopes may be inaccessible due to

TABLE 5. Differentiation of alfalfa mosaic virus (AMV) strains by indirect enzyme-linked immunosorbent assay[a]

	Coating antigen														
	Fixed AMV strain					Native AMV strain					Coat protein of AMV strain				
Antiserum elicited to	H4	N20	S30	S40	W1	H4	N20	S30	S40	W1	H4	N20	S30	S40	W1
Glutaraldehyde-fixed AMV															
H4	0[b]	−	−	−	−	0	−	−	−	−	0	−	−	−	−
N20	−	0	−	−	−	−	0	−	−	−	+	0	−	+	+
S30	−	−	0	−	−	+	+	0	+	+	+	+	0	+	+
S40	−	−	−	0	−	−	−	−	0	−	−	−	−	0	−
W1	−	−	−	−	0	+	+	+	−	0	+	+	+	+	0
Native AMV															
H4	0	−	−	−	−	0	−	−	−	−	0	+	−	−	−
N20	−	0	−	−	−	−	0	−	−	−	+	0	−	+	+
S30	−	−	0	−	−	−	−	0	−	−	+	+	0	+	+
S40	−	−	−	0	−	−	−	−	0	−	−	−	−	0	−
W1	−	−	−	−	0	−	−	−	−	0	+	−	−	−	0
AMV coat protein															
S30 (Rabbit a)	−	−	0	−	−	−	−	0	−	−	−	−	0	−	−
S30 (Rabbit b)	−	−	0	−	−	−	−	0	−	−	−	−	0	+	+
S40 (Rabbit c)	−	−	−	0	−	−	−	−	0	−	−	−	−	0	−
S40 (Rabbit d)	−	−	−	0	−	−	−	−	0	−	−	−	−	0	−

[a] This is a sumamry of tests such as those illustrated in Figure 3.

[b] Tests with homologous strains are indicated by 0 (controls), + indicates that SDI values were 1.0 or greater when calculated (from data such as those shown in Fig. 3) as described by Jeagle and Van Regenmortel (21) and − indicates that SDI rating was less than 1.0.

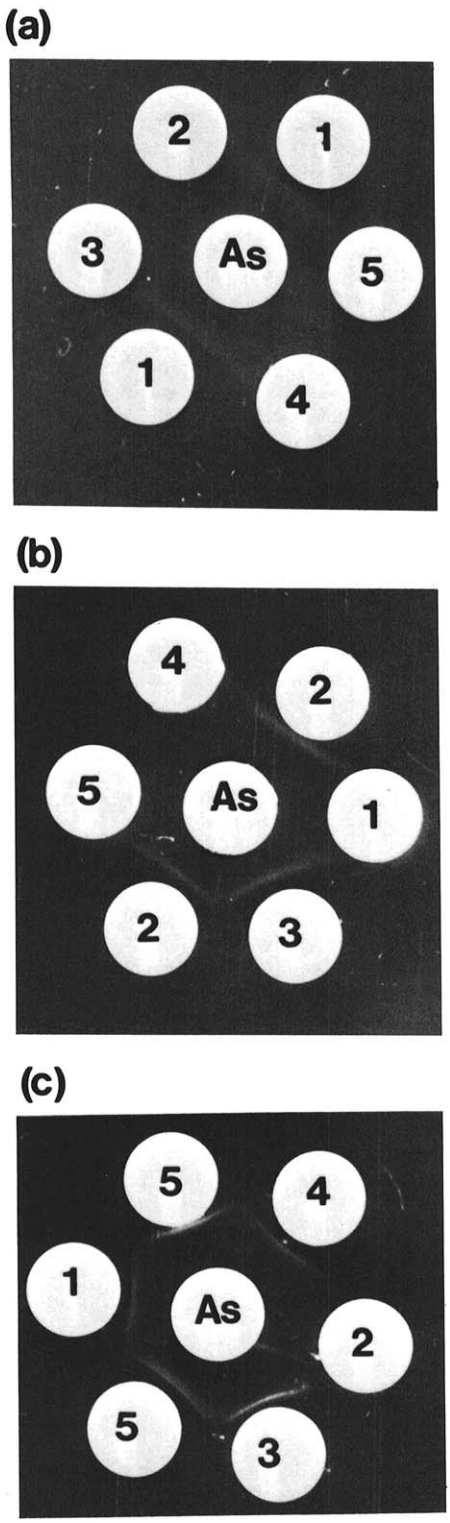

Fig. 4. Reactions of strain-specific or group-specific polyclonal antibodies (As) with glutaraldehyde-fixed virus preparations of AMV-S30 (1), H4 (2), N20 (3), S40 (4), and W1 (5) in agarose gel immunodiffusion tests. Antiserum to native virus preparations of AMV-S30 cross-absorbed with a mixture of glutaraldehyde-fixed virus preparations of all the other heterologous strains was loaded in the central well of **A**; an antiserum to native AMV-H4 after cross-absorption with a mixture of glutaraldehyde-fixed virus preparations of S30, W1, and S40 strains in the central well of **B**. Antiserum to native AMV-W1 after cross-absorption with glutaraldehyde-fixed virus of AMV-H4 was loaded in the central well of **C**. All virus preparations were adjusted to 200 μg/ml. Each well was charged with 10 μl of the appropriate reagent.

adsorption of the antigen to the polystyrene surface (35). Whereas dissociated or partially dissociated AMV particles could selectively mask some of their epitopes in this way, intact virus particles stabilized by fixation would not.

It has been shown with a number of viruses that their antigenic relationships appear to be closer when coat protein subunits rather than intact virus particles are compared (8,30,32,33). It was therefore surprising to find that in ELISA, strain-specific differences of AMV were more readily revealed when isolated coat protein subunit preparations were used as test antigens (Table 5). There are several possible explanations for this. Firstly, it is possible that AMV does have strain-specific cryptotopes that are not, however, detected by immunodiffusion because of the insensitivity of the test. Secondly, it may be that in ELISA, adsorption of the coat protein subunits to the polystyrene is such as to maximize exposure of strain-specific epitopes (cryptotopes and/or metatopes) to antibodies. Thirdly, the antigenic differences observed may be a reflection of the degree to which the coat protein has undergone $CaCl_2$-induced proteolysis. It has been reported that the degree of proteolysis in $CaCl_2$ varies with the virus strain (16). It has also been shown that proteolysis of coat proteins of some viruses can modify their antigenicities (9,14). Indeed, we recently demonstrated the loss of an epitope due to $CaCl_2$ induced proteolysis of AMV coat protein (14), and a similar loss has been reported with some potyviruses (17).

Comparisons of the AMV strains by using antisera elicited to native virus preparations and glutaraldehyde-fixed virus as test antigen showed some nonreciprocal antigenic differences between some of the strains, both in immunodiffusion tests and ELISA. Similar lack of serological reciprocity has been reported with strains of a number of other viruses (3,39,45). It has been suggested that this indicates a lack of specific epitopes on the heterologous antigens or that the epitopes are immunologically silent (45). However, it can also be due to the variation in the response of individual animals to the antigen (41). As we have used antisera from one or at the most two animals for the production of antisera to each of the antigens, it would be hazardous to draw any definite conclusions about the significance of the observed nonreciprocal antigenic relationships between some of the AMV strains.

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