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Serological Relationship of Strains of Tobacco Necrosis Virus and their Ability to Activate Strains of Satellite Virus

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SUMMARY

Antisera prepared in rabbits against strains of tobacco necrosis virus (TNV) or satellite virus (SV) were most specific in precipitation tube tests when animals were bled after single intravenous injections. In Ouchterlony tests, antisera remained equally specific after further injections, including one intramuscular injection. However, all antisera and both test methods agreed in placing the eight strains of TNV tested in two distinctive groups or serotypes. The three strains of SV tested differed as much antigenically from one another as did strains of the two serotypes of TNV, although SV_1 and SVc were more closely related to each other than to SV_2.

Some strains of TNV aid the multiplication of SV_1 and SV_2 but not of SVc; others aid SVc but not SV_1 or SV_2. The ability of different strains of TNV to aid the multiplication of the three strains of SV is correlated with their ability to infect tobacco and bean plants, but not their serological relationship.

INTRODUCTION

Babos & Kassanis (1963) found that seven different strains of tobacco necrosis virus (TNV) could be divided into two groups or serotypes, which they labelled A and D. Antisera had similar precipitation end points when titrated against homologous or heterologous strains of the same group but differed by factors of 8 to 32 when titrated against strains in the other group. Also when an antiserum was adsorbed with a strain of the same group, all or nearly all of the antibody activity was removed, whereas the precipitation end point was unchanged when the antiserum was adsorbed with a strain of the other group. In contrast, Uyemoto, Grogan & Wakeman (1968) failed to find consistent differences when antisera to several strains of TNV were titrated against the homologous and heterologous virus strains, although these included some of groups A and D. The specificity of antisera to some plant viruses changes during the immune response (Koenig & Bercks, 1968; Allen, 1968), and one aim of our work was to see whether such changes could explain the reported differences in serological behaviour of TNV. Another aim was to test the ability of TNV strains to activate strains of satellite virus (SV), and to relate this to serological relationships or other relevant properties.

The subculturing of single local lesions provides pure cultures of TNV from natural mixtures with SV, although Uyemoto *et al.* (1968) failed to achieve this with natural mixtures originating in the U.S.A. Uyemoto & Grogan (1969) have now succeeded, but only after disrupting the viruses with phenol, centrifuging solutions of the mixed nucleic acid, and using the fraction containing the TNV-RNA as inoculum. We have now reinvestigated two of the American mixtures for isolation from those of pure TNV culture.

METHODS

Tobacco necrosis virus inocula. B, A, S (bean stipple streak), E and D are SV-free cultures of TNV previously used at Rothamsted. The cultures AC 43 and AC 36, originally isolated by Fulton (1950), contain SVB and SVC, respectively and were provided by Drs J. K. Uyemoto and R. G. Grogan. Culture GV, provided by Dr M. H. V. van Regenmortel, was isolated from grapevine leaves in South Africa (Cesati & van Regenmortel, 1969). Culture CT was isolated from leaves and flowers of citrus trees in France, and provided by Dr J. Bové.

Satellite virus inocula. SV_1 was propagated in a mixed culture with TNVB, SV_2 with TNVA, SVB with TNVAC 43 and SVC with TNVAC 36.

Plants. TNVS was propagated in French beans (*Phaseolus vulgaris* L. var. The Prince), TNVA, B, E, AC 43 and CT in tobacco (*Nicotiana tabacum* L. White Burley, var. Judy's Pride) and strains TNVD, AC 36 and GV in *Nictoniana clevelandii* Gray. The plants were grown with ample nutrients in a peat-sand mixture free from the fungus *Olpidium brassicae* Dang. and the viruses it transmits.

Virus purification. TNV was alternately (three or four times) precipitated with ammonium sulphate (0·25 g./ml. of clarified sap) and sedimented by ultracentrifugation. The virus was resuspended in distilled water, except after the last ultracentrifugation, when it was resuspended in 0·2 M-sodium acetate buffer, pH 4·5, in which most of the remaining plant protein was insoluble. Residues of plant protein became insoluble during dialysis against distilled water and were removed by centrifugation.

The mixtures of TNV and SV were purified as before (Rees, Short & Kassanis, 1970). Non-infective preparations of SV_1 and SV_2 were obtained by single sucrose density gradient centrifugations of the purified mixtures, but with SVC at least two centrifugations were needed to remove all contaminating TNV. This was because the TNVAC 36 particles survived the purification procedure and were as concentrated as SVC, whereas SV_1 and SV_2 were much more concentrated than TNVA and B, which were eliminated during purification.

Immunization and serological studies. Table 2 shows the different immunization schedules followed. Rabbits were injected each time with 1·5 mg. of purified virus in 1 ml. of saline. The viruses were emulsified in Freund's complete adjuvant for intramuscular injections. The rabbits were bled before each injection. The serological tests were made either in narrow tubes, with purified antigen at a constant concentration of 20 μg./ml. and twofold dilutions of antiserum, or by double diffusion gel-precipitation tests in 0·5% (w/v) Oxoid Ionagar No. 2 in 0·01 M-phosphate buffer, pH 7, containing 0·02% (w/v) sodium azide. We use the ratio of the homologous to heterologous titre to show the degree of specificity of an antiserum. Titre is defined in this paper as the reciprocal of the dilution end point.

Interaction of tobacco necrosis virus and satellite virus strains. These were studied by inoculating an artificial mixture containing 5 μg./ml. of TNV and SV to leaves of *N. clevelandii* dusted with carborundum. The next day the leaves were detached and placed on moist blotting paper in enamelled trays kept in air-tight polythene bags. About 5 days later the sap was extracted, clarified by centrifugation, heated for 10 min. at 50° and centrifuged again at 10,000 rev./min. The concentration of virus in the clarified sap was estimated serologically by titrating the sap against antiserum used at a dilution that ensured specificity.

RESULTS

Infectivity and symptoms of tobacco necrosis virus strains

The susceptibility of tobacco and French bean plants to different strains of TNV differs greatly. Strains producing few lesions also produce small lesions containing very little virus, and these were therefore propagated in *Nicotiniana clevelandii*. Table 1 groups the strains of virus according to transmissibility to tobacco and beans and to the type of symptoms produced. Except for TNVs, which infects systemically and does not form typical necrotic lesions, the necrotic local lesions produced in French bean plants are of three types: (1) lesions that spread to form a necrotic net when there are only a few lesions

Table 1. *Comparison of infectivity and symptoms of TNV strains*

TNV strain	Type of local lesion on French bean	Infectivity on tobacco and French bean
S	No definite local lesions, systemic infection	Easy to infect
A, B	Large spreading necrotic local lesions forming necrotic nets	As above
E, AC 43, CT	Restricted angular necrotic local lesions slightly spreading	As above
D, AC 36, GV	Round necrotic nonspreading local lesions, often minute	Difficult or impossible to infect

Table 2. *Comparison of homologous and heterologous titres at different stages of the immune response and immunization schedules*

	Rabbit 18			Rabbit 19	
Injection and bleeding time (weeks)	Homologous (TNVA)	Homologous/ heterologous (TNVD)	Injection and bleeding time (weeks)	Homologous (TNVA)	Homologous/ heterologous (TNVD)
0	Intravenous	—	0	1st Intravenous	—
1	256	64	2	128	16
2	512	64	2	2nd Intravenous	—
4	256	32	4	1024	16
7	128	8	5	1024	16
10	128	16	7	1024	32
10	Intramuscular	—	9	256	16
11	256	4	14	64	4
12	1024	4	19	32	4
14	1024	4	26	32	4
18	2048	4			
26	1024	4		(TNVD)	(TNVA)
	Rabbit 20			Rabbit 24	
0	1st Intramuscular	—	0	1st Intravenous	—
2	256	16	1	128	16
4	512	16	2	128	16
6	512	8	3	64	16
7	512	8	3	2nd Intravenous	—
9	512	7	5	2048	16
9	2nd Intramuscular	—	6	2048	16
	Rabbit died		10	256	8
			10	Intramuscular	—
			14	2048	4
			16	2048	4

per leaf; (2) lesions which are restricted but angular because they spread slightly into minor veins; (3) lesions which are restricted, round and often minute.

The strain TNVGV, which does not infect tobacco, was received as dried *Chenopodium quinoa* Willd. leaves, which readily infected *Chenopodium amaranticolor* Coste & Reyn. but not tobacco, French bean or *N. clevelandii*. As sap of *Chenopodium* species inhibits strongly the infection of other plants by viruses, other plants were infected only by using as inoculum a phenol extract of infected leaves. TNVGV infected *N. clevelandii* with ease, French bean with difficulty and tobacco plants not at all.

Serological relationships between tobacco necrosis virus strains

Table 2 shows the homologous titres of sera of different immunization schedules and the ratios of homologous to heterologous titres. The differences between homologous and heterologous titres depended on the immunization schedules. Two weeks after one intravenous or intramuscular injection the primary response was weak (homologous titres 128 to 512), but the sera were specific (homologous titre/heterologous titre = 16). The titres increased after the secondary response, but whereas specificity remained unchanged after another intravenous injection, it decreased after an intramuscular injection (the titre ratio dropped from 16 to 4). When one intramuscular injection followed two intravenous injections (rabbit 24) specificity also declined. With all immunization schedules the specificity of the serum declined with delay in bleeding. Antisera with maximum titres were not necessarily the least specific and there was no correlation between levels of homologous and heterologous titres. Similar results, not included in Table 2, were obtained with an antiserum to TNVAC 36.

Table 3. *The specificity of antisera to different strains of TNV as affected by the immunization schedule*

	Serological titres			
	Antisera* to TNVA		Antisera to TNVD	
Test antigen at 20 μg./ml.	Early	Late	Early	Late
TNVA	256	4096	8	512
TNVS	256	4096	8	512
TNVE	4	1024	128	2048
TNVAC 43	4	1024	128	2048
TNVCT	4	1024	—	—
TNVD	4	1024	128	2048
TNVAC 36	4	1024	128	2048
TNVGV	4	1024	—	—

* 'Early' antisera were obtained 1 week after one intravenous injection. About 10 weeks later when the serological titres of the antisera were declining, the rabbits received an intramuscular injection and 2 weeks later the 'late' antisera were obtained (Table 1).

In grouping the virus strains a constant amount of each antigen was titrated against the most and least specific antisera to TNVA and TNVD. The serological grouping of the different strains (Table 3) is unequivocal whichever type of antiserum is used. Similar results were obtained using an antiserum to TNVAC 36. Serological titres are the same for all virus strains within the same group. The differences between titres for strains belonging to different groups were greater when using an antiserum from an early bleeding in the primary response than for antiserum from a late bleeding in the secondary response. The results obtained with primary response antisera resemble those of Babos & Kassanis (1963) who used antisera prepared by two intravenous injections one week apart and collected 10 days later.

Similar results were obtained in double-diffusion gel tests when undiluted antisera to

TNVA or D were placed in the central wells and the different strains of virus at 1 mg./ml. in surrounding wells. Virus strains of the same group as that used to produce the antiserum developed either small spurs or none, whereas virus strains of the heterologous group developed large spurs. Spurs of similar length were obtained with antisera from the primary and secondary immune responses, but the band obtained with heterologous strains of virus was as intense as that produced when the homologous virus reacted with antisera of the secondary response (Fig. 1 *a–d*).

Serological relationships between satellite virus strains

The three virus strains SV_1, SV_2 and SV_3 were isolated at Rothamsted and differ considerably in antigenic properties (Kassanis, 1966; Rees *et al.* 1970). SV_3 only multiplies well in roots of plants infected with the fungus *Olpidium brassicae* and was not used since it is difficult to propagate in leaves of tobacco plants. Of the three strains described in the U.S.A. (Uyemoto *et al.* 1968), SVA originated at Rothamsted and is identical to SV_1; SVB was found to be serologically identical to SV_2. In contrast, SVC differs from the English strains. An antiserum against SVC was prepared by one intravenous injection followed 2 weeks later by an intramuscular injection. Serum taken after the first injection had a titre of 128 when tested with SVC and titres of 8 and 4 respectively against SV_1 and SV_2. Twelve weeks after the intramuscular injection the titre with SVC was 512, and to SV_1 and SV_2 the titres were 512 and 128, respectively. Thus, as with TNV strains, the antiserum to SVC taken after an intravenous injection followed by an intramuscular injection was less specific. Gel-diffusion tests showed that SVC and SV_1 have a common antigenic determinant not present in SV_2, and that SV_C also has an antigenic determinant which the other two strains do not have. These differences appeared when using antiserum taken after the intramuscular injection, because antiserum taken after one intravenous injection failed to react with SV_2 (Fig. 1 *e,f*). Although the differences between the serological titres with SVC and SV_1 disappeared after intramuscular injection, the spurs formed in gel-diffusion tests were of the same length.

Separation of tobacco necrosis virus from natural mixtures with satellite virus

Unlike Uyemoto *et al.* (1968), we obtained without difficulty a pure culture of TNV from a natural mixture of TNVAC 36 and SVC which contained about the same amount of the two viruses. Sap was diluted so that it produced only a few lesions per leaf in *N. clevelandii*, and after seven single lesion transfers the culture contained only TNV. The natural mixture TNVAC43 and SVB contains considerably more SV than TNV, and for this reason TNV was separated first from a purified preparation of the mixture by centrifugation in a sucrose density gradient before use as inoculum to produce local lesions from which inocula were taken for serial single lesion isolations. Pure cultures of AC 36 and AC 43 were maintained free from SV after seven bulk transfers; American workers have adopted this test for purity. Cultures of some TNV strains can be readily contaminated with SV. Thus, TNVD does not activate any of the Rothamsted strains of SV, and less stringent methods are required when this strain is cultured. Two months after obtaining SVC from the U.S.A., it was found as a contaminant in our TNV culture. This contamination is best prevented by placing prototype inocula as small samples at $-15°$ as soon as the culture is pure; continuous reculturing in growing plants is hazardous. Cultures of TNVA, TNVB and TNVS isolated some years ago from natural mixtures remained free from SV during many subcultures. It is of interest to mention that strain TNVS differs from the rest in that it infects French beans systemically and can be isolated from a natural mixture by taking inocula serially from young trifoliate leaves as soon as they become systemically infected (Kassanis, Vince & Woods, 1970).

Activation of different strains of satellite virus by strains of tobacco necrosis virus

None of the Rothamsted strains of SV is activated by TNVD but Uyemoto *et al.* (1968) found that SVC was activated by TNVD and by three other strains of TNV. They also found that

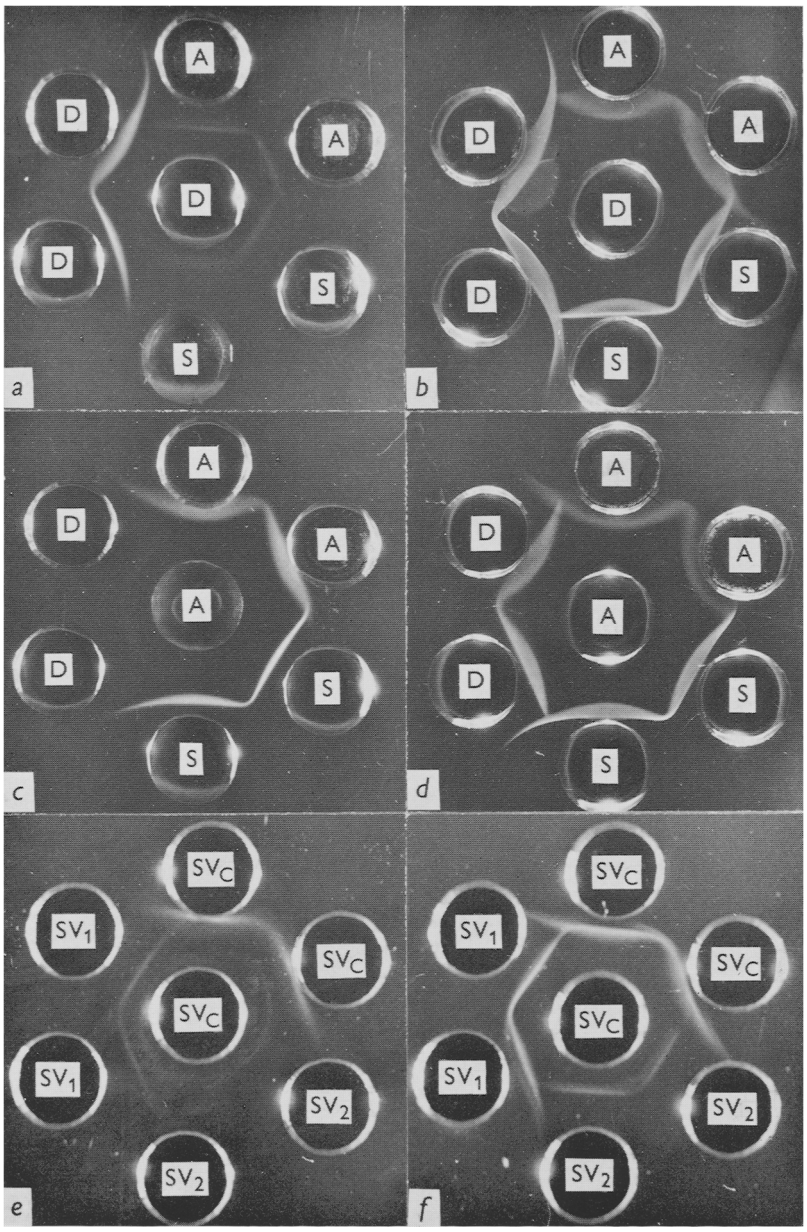

Fig. 1. Ouchterlony tests with strains A, D and S of TNV (*a* to *d*) and strains of SV (*e*, *f*). Antisera are in the central wells and were taken early in the primary immune response after one intravenous injection (*a*, *c*, *e*) or late in the secondary response from the same rabbits after an additional intramuscular injection (*b*, *d*, *f*). Antisera were undiluted except in the gel shown in *f* where the dilution was 1/5. The antigens in the peripheral wells were at a concentration of 1 mg./ml. for TNV strains and 0·5 mg./ml. for SV strains.

the strains that activate SVc do not activate SVA or SVB and *vice versa*; our results confirm this (Table 4). There is no correlation between the serological affinity of TNV strains and their ability to activate the three SV strains, except possibly that strains of group A activate SV_1 and SV_2. However, the ability to infect tobacco and French bean plants (Table 5) seems to be correlated with the ability to activate SVc. Because SVc is activated by strains of TNV that are either difficult or impossible to transmit to these plants, the strain of TNV recently isolated from diseased pear and apple trees should also activate SVc (Kegler *et al.* 1969). Antigenically, SVc is closer to SV_1 than to SV_2, although SV_1 and SVc are activated by different TNV strains. Table 4 also shows that SV inhibits the multiplication of TNV; the extent of inhibition depends on the particular combination of viruses and possibly on the species of plant (Babos & Kassanis, 1963). Except for strain TNVCT, the inhibition is considerable with SV_1 and SV_2 and negligible with SVc.

Table 4. *Activation of three strains of SV by nine different strains of TNV*

Strain of TNV used for activation	Titres of SV			Titres of TNV in mixtures and when inoculated alone			
	SV_1	SV_2	SVc	$+SV_1$	$+SV_2$	+SVc	Alone
TNVA	32	32	0	2	4	32	32
TNVB	64	64	0	8	8	32	32
TNVS	32	32	0	8	8	64	64
TNVE	32	32	0	8	8	32	64
TNVAC 43	64	32	0	4	16	64	64
TNVCT	64	16	0	16	16	32	32
TNVD	0	0	16	32	32	16	32
TNVAC 36	0	0	64	32	32	32	64
TNVGV	0	0	64	32	32	32	64

Table 5. *Grouping of TNV strains on serology, activation and infectivity on tobacco and French bean plants*

Strain of TNV	Serological group	SV strain activated	Infectivity on tobacco and French bean
A, B, S	A	SV_1 and SV_2	Easy to infect
E, AC 43, CT	D	SV_1 and SV_2	Easy to infect
D, AC 36, GV	D	SVc	Difficult or impossible to infect

DISCUSSION

We found no difficulty in determining the serological grouping of different TNV strains. However, in tube tests the greatest antigenic difference between the two groups was found with antisera obtained during the primary immune response. The failure of Uyemoto *et al.* (1968) to separate the strains antigenically into the two groups is perhaps due to their use of antisera which were probably non-specific, because of the injection and bleeding schedules used.

The lack of correlation between the serological affinity of strains of TNV and their ability to activate strains of SV is interesting. Strains closely related serologically usually have other properties in common, although this is not the case with strains of the D group which activate different strains of SV. However, the ability to activate SV seems to be correlated with host range, and those viruses which multiply readily in tobacco and French bean activate different SV strains from those activated by virus strains which multiply with difficulty in these plants.

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