Improvement of Arbovirus HA Antigens by Treatment With a Colloidal Silica Gel and Sonication

By

T. TRAAVIK

Virus Department A, National Institute of Public Health,
Oslo, Norway

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Summary

A remarkable increase in HA titers for weakly haemagglutinating Norwegian arbovirus strains, Uukuniemi and Runde viruses, was achieved by including treatment with the colloidal silica gel Aerosil in the antigen preparation scheme. By combining this procedure with sonication, the titers of sucrose-aceton extracted, infected suckling mouse brains could be increased several hundred times. Good antigens also were obtained from virus grown in BHK 21/c13 cell cultures and concentrated by polyethylene glycol 6000/NaCl. Rubella virus HA antigen and HB_sAg were adsorbed to the gel, and excluded from a preparation by treatment with Aerosil. This indicates a limitation to the universal use of the method, presumably related to the particle size.

Introduction

Due to speed, sensitivity, economy and simplicity the haemagglutination inhibition test (HAI) has been a standard test in arbovirus serology. This applies to both identification of virus isolates and serological surveys and diagnostics.

For production of arbovirus haemagglutinating (HA) antigens, the classical sucrose-aceton extraction method (SA) (3) with infected suckling mouse brains has been almost undisputed, although methods based on infected cell culture fluids (2, 12) and infected mouse ascites fluids (8) have also been reported.

The arbovirus strains isolated in Norway up to date (15, 16, 17, 18), have all presented modest yields of HA antigens by the conventional SA method. Tween-ether treatment (9) has not increased the titers appreciably.

The low titers of suckling mouse brain preparations necessitate a high consumption of mouse litters and frequent performance of the rather cumbersome and laborious SA method. For unknown reasons, variations in the quality of the antigen from batch to batch are adding to the difficulties.

Consequently, there is a marked need for a reliable and simple method to prepare high-titered HA antigens from infected tissues or cell cultures.

Since the haemagglutination inhibitors in virus preparations appear to be of lipoprotein nature (4, 6, 11), a procedure for HA antigen production might be based on a compound which removes lipoproteins without interference with the quantity or configuration/composition of the genuine HA antigens.

The colloidal silica gel "Aerosil" has demonstrated capability to adsorb serum lipoproteins and also the hepatitis B antigen (HB_sAg) including the Dane particle (13, 14). It seemed worthwhile to investigate whether this compound would adsorb inhibitors to HA, leaving the viruses/HA antigens in solution. Since a beneficial effect of sonication on arbovirus HA titers has been reported (1), it was decided to include ultra sound treatment into the investigations.

Materials and Methods

Virus Strains

The strain SF E 1 was isolated from engorged *Ixodes ricinus* ticks (nymphs) collected from migrating passerine birds captured at Store Føerder (an island in the Oslofjord) in May 1973. By HAI it was demonstrated to be serologically related to the S23 prototype Uukuniemi virus and that it also had the same morphology (15, 17).

The strain Ru E81 was isolated from unengorged *Ixodes uriae* (females) collected in the seabird colony at Runde in September 1973. In the electron microscope it presents a morphology which closely resembles that of the corona virus group (16, 18).

A Tween-ether extracted Rubella HA antigen from Behringwerke has also been used in these investigations.

Antigen Preparations

Crude suckling mouse brain preparations (SMB antigens) consist of approximately 20 per cent suspensions of infected brain material in borate saline pH 9.0 or in PBS pH 7.4. SF E 1 was used at the fourth mouse brain passage, titring 7.2 logs₁₀ BMLD₅₀/ml (baby mouse lethal doses). The fifth SMB passage of Ru E81 which titred 6.1 logs BMLD₅₀/ml constituted the antigenic source for this virus. To get rid of tough debris, the brain suspensions were centrifuged once at 5000 × *g* for 15 minutes.

Sucrose-aceton extracted suckling mouse brains (SA antigens) were prepared according to CLARKE and CASALS (3), but the final lyophilization step was omitted.

Virus precipitates from cell culture fluids (BHK antigens) were prepared as follows: Polyethylene glycol (PEG) 6000 (Macrogolum 6000, Norsk Medicinaldepot), 6 g/100 ml, and NaCl, 2.2 g/100 ml, were dissolved in culture fluids harvested from virus infected BHK 21/c 13 cultures (7). The pH was adjusted to 7.6, and precipitation took place at 4° C overnight. On the following morning the culture fluids were centrifuged at 5000 × *g* for 20 minutes. The supernates were discarded, and the precipitates redissolved in PBS with 0.75 per cent bovine serum albumine or borate saline with 0.4 per cent albumine (BABS). The antigens used in these experiments were made by redissolving precipitates in 1/300 of the original volume.

The strains employed in these experiments had the following passage history: SF E 1 had passed 5 times in mice, and thereafter undergone 6 to 9 passages in cell culture. Ru E81 was used after 2 mouse brain passages and 5—8 passages in BHK 21/c 13 cells. The rubella HA antigen was used as such.

Absorption of Inhibitors

Aerosil 380 (Degussa, Frankfurt a. Main), according to the manufacturer is a colloidal silica consisting of aggregated 7 nm particles with a surface area of about 380 m²/gram.

Aerosil Adsorption

This was performed as follows: The antigen solutions were mixed with 10, 20, 40, 60, 80 and 100 mg Aerosil per ml. The mixtures were treated with a swirler (Cenco

Instrumenten, Breda, the Netherlands) to ensure a homogenous suspension to be effected. The tubes were placed in a waterbath with automatical shaking (type 3047, J. Köttermann, K.G. Hanigsen, Germany) for 2 hours at 45° C and centrifuged for 30 minutes at 5000 $\times g$. The antigen-containing supernate was pipetted off and kept, while the sedimented silica gel was discarded.

Sonication

A Branson B12 sonifier (Branson Sonic Power S.A.) with microtip was employed. For cooling, an ethanol-wet ice mixture was used. Only 2 time/power combinations have been investigated this far:

1. Three bursts at 100 W for 15 seconds.
2. A continuous treatment at 60 W for 3 minutes.

Haemagglutination and Haemagglutination Inhibition Tests

HA and HAI tests were performed essentially as described by CLARKE and CASALS (3), modified for microtitration equipment (Cooke Eng. Co., Alexandria, Virginia) and using chicken erythrocytes instead of the recommended goose cells. The HA activity was tested with the following antigen preparations of each virus parallelly:

- a) Untreated; b) Aerosil-treated; c) Sonicated; d) Aerosil-treated and then sonicated; e) Sonicated and then Aerosil-treated.

Uninfected suckling mouse brains, SA extracted suckling mouse brains and culture fluids from uninfected BHK 21/c13 cultures had been treated as the antigens and were included in every test as controls. HA activity was investigated in the pH range of 5.6—7.4 at +4° C, room temperature and 37° C.

In order to confirm the virus specificity of observed HA reactions, specific mouse antisera, and a serum pool from uninfected mice were utilized in HAI. Six units of antigen were employed in these tests.

Titration of Infectivity and Precipitins

Infected brain suspensions and PEG treated cell culture concentrates were titrated by intracerebral inoculation in suckling mice after Aerosil absorption, incubation with shaking in waterbath at 45° C and untreated.

Precipitin activity was titrated by immunoelectroosmophoresis in 1 per cent agarose gel as described elsewhere (17, 18).

Results

Optimal Concentration of Aerosil

By employing 10 mg Aerosil per ml, the HA titers were increased from 4- to 8-fold, depending on the kind of antigenic preparation, for SF E1 and Ru E81. By using 20 mg silica gel per ml, a further 4- to 8-fold increase was obtained. No beneficial effect was recognized by increasing the concentration above this level. Accordingly an Aerosil concentration of 20 mg/ml was chosen throughout the experiments.

The rubella HA antigen initially titered 256—512. This was reduced to 32 to 64 after treatment with 10 mg Aerosil per ml and to nil when 20 mg/ml was used. At 80 mg/ml some HA activity appeared. This may be due to inefficient mixing. These results are summarized in Table 1.

Dependence on pH and Temperature

SF E1 virus was earlier shown to have a marked pH dependence in the HA reaction, with maximum activity at pH 5.6—5.8 for SA preparations. The pH effect on Ru E81 was not as manifest, nearly constant HA titers being obtained

at pH 6.2—7.2. After Aerosil treatment the pH spectrum for HA was broadened, although SFE1 still possessed a maximum at pH 5.6—6.2 (Table 2).

Table 1. *The effect of increasing Aerosil concentration on HA titers (reciprocal values)*

Antigens	HA titers after treatment with (mg/ml)						
	0	10	20	40	60	80	100
SF E1 SA	32	512	2048	2048	1024	512	1024
E1 SMB	2	8	32	32	16	16	8
E1 BHK	8	16	128	128	64	64	64
Ru E81 SA	128	1024	2048	1024	1024	1024	512
E81 SMB	16	64	64	128	64	32	64
E81 BHK	32	128	512	512	1024	256	256
Rubella ^a	256	32	0 ^b	0	0	2	4

^a Rubella HA antigen, Behringwerke Batch F-170

^b No haemagglutination undiluted

Table 2. *The influence of Aerosil treatment (20 mg/ml) on the pH dependence of HA reactions*

Antigens	HA titers ^a at pH							
	5.6	5.8	6.0	6.2	6.4	6.8	7.0	7.4
SF E1 untreated	64	64	32	16	8	2	2	0
E1 Aerosil	1024	2048	1024	256	256	64	64	16
Ru E81 untreated	16	16	32	64	128	256	64	32
E81 Aerosil	512	1024	2048	1024	4096	2048	1024	512

^a HA titers in reciprocal values

The incubation temperature had earlier been shown to affect the HA of SF E1 severely. The highest titers were obtained at 37° C, while nearly no HA activity was recorded at 4° C. The influence of varying temperatures was less for Ru E81, but somewhat higher titers and clearer end-points were obtained at 4° C.

After absorption with silica gel, SF E1 haemagglutinated at 4° C, although titers were lower than at 37° C. For Ru E81 titers were essentially the same at 4° C and 37° C, but sedimentation patterns were better at the lower temperature (Table 3).

Sonication

No differences were recorded between the two sonication procedures applied. In some instances a 2- to 4-fold titer increase was registered, but at other times HA titers were unaffected by sonication alone (Table 4).

The Effect of Aerosil on Infectivity and Precipitins

It was shown, as demonstrated in Table 5, that the drop in infectivity titer seen for Ru E81 was mainly due to the incubation at 45° C. SF E1 was unaffected by Aerosil treatment. Precipitin titers were not affected for any of the viruses. But

the results were easier read after Aerosil treatment, because lipoprotein deposition around the wells, which is sometimes a problem, was avoided.

Table 3. *The influence of Aerosil treatment on the optimal incubation temperature for HA reactions*

Antigens	HA titers ^a at	
	4° C	37° C
SF E1 untreated	0	64
E1 Aerosil	256	2048
Ru E81 untreated	128	128
E81 Aerosil	2048	1024

^a HA titers in reciprocal values

Table 4. *The influence of sonication^a on HA activity of antigens not treated by Aerosil*

Antigens	HA titer ^b of the preparation	
	Untreated	Sonicated
SF E1 SA	32	64
E1 SMB	2	2
E1 BHK	8	32
Ru E81 SA	128	128
E81 SMB	8	16
E81 BHK	32	64

^a Three bursts a 100 W for 15 seconds

^b HA titers in reciprocal values

Table 5. *The influence of Aerosil treatment on the infectivity to suckling mice and the precipitin titers^a of SF E1 and Ru E81 virus strains*

Virus strain	End points in log ₁₀ BMLD ₅₀ ^b			Precipitin titers		
	Controls	Inc. ^c	Aerosil	C	I	A
SF E1	6.2	5.9	5.8	32	32	64
Ru E81	5.8	3.2	2.8	16	16	16

^a Baby mouse lethal doses₅₀ by intracerebral inoculation

^b Titers in reciprocal values

^c Incubation at 45° C for 2 hours

Final Results

For SF E1 compared titrations were performed at 37° C with final pH 5.8, for Ru E81 the parameters were 4° C and pH 6.4. Absorption was executed with 20 mg Aerosil per ml and sonication was performed with 3 bursts a 15 seconds and 100 W output.

The results are summarized in Table 6. There was no obvious difference between performing sonication before or after Aerosil treatment, but the combination of these two procedures proved valuable. As can be seen the highest titers were obtained with SA extracted mouse brain preparations. But fairly acceptable and useful HA antigens can be produced from cell culture fluids and crude suckling mouse brains.

The HA reactions observed are virus specific as demonstrated by homologous serum inhibitions and negative reactions for control preparations.

Table 6. *The influence of combinations of Aerosil treatment (A) and sonication (S) on HA activities*

Antigen	HA titers ^a after treatment with				
	U ^b	S	A	A + S	S + A
SF E1 SA	32	64	2,048	4,096	8,192
E1 SMB	2	4	32	64	64
E1 BHK	8	32	128	1,024	512
Ru E81 SA	128	256	2,048	16,384	8,192
E81 SMB	16	16	128	256	256
E81 BHK	32	128	512	1,024	2,048

^a HA titers in reciprocal values

^b Untreated

Discussion

The experiments described have demonstrated the ability of the colloidal silica gel Aerosil to absorb supposed HA inhibitory factors from virus antigen preparations. The two viruses investigated are large, 80–220 nm in diameter (15, 16, 17, 18). The results obtained with Tween-ether extracted rubella virus and with HB_sAg (13), when Aerosil actually absorbs the virus specific antigens, points to a limitation to the method, presumably presented by the size of the virion. The exact limit is not known at present, but the data available indicate that the critical diameter is somewhere between 50 and 80 nm. It seems unlikely that the Aerosil absorption method can be applied to the smaller alpha and flaviviruses.

Methods used in the past to improve the capability of low-titered HA antigens or to bring non-haemagglutinating viruses to react, have been based on detergents (9, 10) or enzymes (1, 5). Reaction conditions must be carefully controlled with regard to concentration and time in order to obtain satisfactory results. Since the effect of Aerosil seems to be due to a simple absorption effect without any interference with chemical composition or configuration, the problems mentioned are eliminated.

The results obtained with virusinfected cell culture fluids after precipitation with PEG 6000/NaCl and Aerosil absorption imply that antigens of this kind represent a useful alternative to the conventional suckling mouse brain antigens. The inferiority in HA titers will be more than compensated by the gain in resources and economy and the simplicity of production.

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Author's address: Dr. T. TRAAVIK, Institute of Medical Biology, University of Tromsø, 9001 Tromsø, Norway.

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