Application of the Immunofluorescent Technique in Testing the Purity of Sendai Virus

By

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Various attempts have been made in this laboratory to eliminate the host components surrounding Sendai virus particles. Procedures such as fluorocarbon treatment, sonication and methanol precipitation resulted in the loss of infectious titer of the test materials. However adsorption on and elution from chicken red cells or aluminum phosphate gel did not cause loss of infectious titer.^{1,2,3)} Because of the hemolytic properties of Sendai virus when propagated *in ovo*, aluminum phosphate gel was chosen for purification purposes. Fundamental studies on this technique have been reported in the preceding paper.⁴⁾ Highly purified preparations with regard to their hemagglutinin titers per mg nitrogen, showing a single component on ultracentrifugation, can be reproducibly obtained in this manner. The serological analysis of these preparations was then attempted.

One of the serological procedures used for analysis of such preparations is the agar gel precipitin test, which has the advantage of differentiating various antigens.^{5,6,7)} Another method is the immunofluorescent technique, which is a simple test of purity during purification. The present paper is chiefly concerned with the development of a immunofluorescent spot technique along the latter line using antibodies prepared against normal sedimentable materials.¹⁾

MATERIALS and METHODS

Virus: The Fushimi strain of Sendai virus, was used. The history and propagation procedures have been reported previously.⁴⁾ In the present work, allantoic fluid cultures obtained by 72 hours incubation at 36°C were used, after centrifugation at 6,000 rpm for 20 minutes.

Purification of the virus by a combination of differential centrifugation and adsorption on aluminum phosphate gel: Details of the procedure used were described in the previous paper.⁴⁾ The infected allantoic fluid was centrifuged at

43,750 *g* for 30 minutes and the resulting pellet was suspended in 1/10 volume of distilled water and recentrifuged at 6,000 rpm for 20 minutes. The material obtained was designated as sedimented virus. The hemagglutinin (HA) titer of this suspension was 32,000 units/ml. Then 2 grams of aluminum phosphate gel (200 units) was added to 400 ml of the above suspension which containing a total of about 10^6 HA units of virus. After an adsorption period of 10 minutes at 4°C, this was centrifuged at 6,000 rpm for 10 minutes and the supernatant was discarded. The material was eluted four times with 400 ml aliquots of 0.25 M phosphate buffer of pH 8.0. Each eluate was centrifuged at 43,750 *g* for 30 minutes and the pellet obtained was suspended in 4 ml of water and recentrifuged at 6,000 rpm for 20 minutes. The eluates are designated as eluate -1, -2, -3 and -4 in the text. For fluorescent spot staining, each viral suspension was diluted with phosphate buffered saline (PBS) of pH 7.1 and adjusted to 10,000 HA units/ml. The nitrogen contents of these diluted suspension with the sedimented virus, and eluates -1, -2 and -3 were 0.1, 0.07, 0.04 and 0.03 mg N per ml, respectively.

Preparation of normal sedimentable material from non-infected allantoic fluid: Normal allantoic fluid obtained from 14-day-old chick embryos was first centrifuged at 6,000 rpm for 20 minutes to remove the cell debris. Then the supernatant was centrifuged at 43,750 *g* for 30 minutes to precipitate the so-called normal sedimentable materials. The pellet obtained was resuspended in PBS and the differential centrifugation described above was repeated. Finally the pellet was resuspended in 1/50 of the starting volume of PBS. The nitrogen content of this suspension was 0.1 mg N per ml. This is designated as NSP (normal sedimentable protein) antigen in the text.

Preparation of immune serum against Sendai virus: For the preparation of hyperimmune sera, three rabbits were immunized by intravenous injection of 5 ml of infected allantoic fluid twice a week for 3 weeks. Two weeks after the last injection the rabbits were bled and their sera were pooled. This material is designated as unadsorbed anti-viral serum in the text.

Preparation of immune serum against normal sedimentable material: Three rabbits received intravenous injections of 5 ml of normal allantoic fluid obtained from 14-day-old chick embryos twice a week for 4 weeks. Two weeks after the last injection, the rabbits were bled and their sera pooled. This is called anti-NSP sera in the text.

Preparation of normal chorio-allantoic membrane powder: To remove antibodies against normal host components from the anti-viral serum described above, adsorption by the antigen prepared from normal chorio-allantoic membrane (CAM) was attempted. This antigen was prepared as follows: The CAM from four dozen 14-day-old embryonated eggs was washed with four 500 ml changes of PBS. Then the membranes were drained on gauze, quickly washed with distilled

water and deposited on filter paper fitted to a Buchner's funnel. Then twelve membranes were suspended in 10 ml of distilled water. This suspension was frozen and thawed three times. These membranes were then lyophilized and ground in a mortar, without abrasive. The powder obtained was used as antigen for the adsorption.

Preparation of adsorbed anti-viral serum: Anti-viral serum was adsorbed with CAM powder. To do this, the serum was diluted ten times with PBS and 1 ml aliquots of the diluted serum were mixed with 10 mg aliquots of the powder. The mixture was incubated at 37°C for 60 minutes with occasional shaking and then left overnight in the cold. The mixtures were then centrifuged at 15,000 rpm for 60 minutes in a refrigerated centrifuge. This procedure was repeated twice and the resulting supernatant was used as the factor anti-viral serum in the fluorescent spot technique.

Hemagglutinin titration and the hemagglutination inhibition test: For hemagglutinin titration and the hemagglutination inhibition test (HAI), the fractional dilution procedure of Horsfall and Tamm⁸⁾ was employed using a one per cent chicken red cell suspensions.

Titration of hemolytic activity: For titration of the hemolytic activity of the Sendai virus, the procedure described by Sato⁹⁾ was employed. Serial twofold dilutions of the viral material were made with 1 ml aliquots of PBS and to these tubes were added 1 ml aliquots of 2 per cent guinea pig red cell suspensions. After mixing, the tubes were incubated at 37°C for 3 hours, and then centrifuged at 3,000 rpm for 2 minutes. To 1 ml of the supernatant, 9 ml of distilled water was added and the degree of hemolysis was determined in a colorimeter (Hitachi EPO-A) with a blue filter (400–480 m μ). The degree of hemolysis was calculated from a standard solution showing 100 per cent hemolysis. One hemolytic unit was expressed as the reciprocal of the final dilution of viral material showing 25 per cent hemolysis. A standard solution was obtained as follows: 9.5 ml of distilled water was added to 0.5 ml of 2 per cent guinea pig red cell suspensions and this mixture was incubated for 3 hours at 37°C. After light centrifugation, one drop of dil. ammonium hydroxide solution was added to the supernatant.

Complement fixation test: The method of Lennette was employed.¹⁰⁾ The details of this method have been described by Ikeda.¹¹⁾ The system contained 0.2 ml of antigen (2 units), 0.2 ml of antiserum, 0.2 ml of complement (2 exact units) and 0.5 ml of sensitized sheep red cell suspension.

Preparation of complement fixing viral antigen (CF-V): As CF-V antigen, an appropriate dilution of sedimented virus in Kolmer's saline was used.

Fluorescent antibody: The material used was a globulin fraction of goat serum containing antibodies to normal rabbit gamma globulin. The anti-rabbit globulin serum was conjugated with fluorescein isothiocyanate by the method of Marshall *et al.*¹²⁾ It was adsorbed twice with mouse liver powder, before staining.

Fluorescent spot technique: The indirect method of Coons¹³⁾ was used in the present experiments. For the spot technique, the method of Crawford *et al.*¹⁴⁾ was followed. Glass slides of high ultraviolet transmittance were washed twice in acetone and dried. A platinum loop of 1 mm diameter was dipped in the antigen solution, and the antigen solution was then spread over a circular area of 3 mm in diameter on the slide. This material was dried, and fixed for 10 minutes in acetone. Then the area was covered with one drop of the diluted immune serum. The slides were placed in a humidified, air-tight container and agitated with shaker for 30 minutes at room temperature. When the reaction was complete, residual serum was washed off by three changes of PBS at 10 minutes intervals. The slide was dried, and one drop of fluorescent antibody was put on the antigen area and the slide was shaken again for 30 minutes. The slide was then washed with three changes of PBS at 10 minutes intervals and then with distilled water and finally dried.

Fluorescence microscopy and reading of the result: A Carl Zeiss "Ultraphoto" microscope, equipped with a dark field condensor, was used in conjunction with a high pressure mercury vapor lamp, an Osram HBO 200 and a BG 12 filter.

The degree of fluorescence was expressed as follows: —, no fluorescence; ±, a trace of fluorescence; +, slight fluorescence; #, moderate fluorescence; ##, marked fluorescence.

Test of the specificity of the reaction in the fluorescence spot technique: To confirm the specificity of this reaction, control runs were made as follows:

- 1) Saline was used instead of viral and normal antigen.
- 2) Either saline or normal rabbit serum was used in place of the anti-viral or anti-NSP sera.
- 3) Viral and NSP antigens were treated with fluorescent antibody only.
- 4) Normal goat serum was used in place of the fluorescent antibody.
- 5) Viral and NSP antigens were treated with anti-viral or anti-NSP serum, and then with unlabelled anti-rabbit globulin goat serum globulin. After these pretreatments, they were stained with the fluorescent antibody.

None of the control runs showed any fluorescence.

EXPERIMENTAL

1. Examination of viral antigen and normal host component in the purified virus preparation

For detection of viral antigen and normal component by the fluorescent spot technique, the following test samples were used: sedimented virus, eluates-1, -2, and -3 and NSP.

First the sedimented virus was reacted with a 10 fold dilution of unadsorbed anti-viral serum (the CF-V titer of the original serum was 1: 1280) and then treated with the fluorescent antibody. Marked fluorescence was observed (Fig. 1).

However, when the preparation was stained using anti-NSP serum, moderate fluorescence appeared (Fig. 2). Therefore the sedimented virus may still contain host components which react with the anti-NSP serum.

When a NSP antigen suspension was stained, using unadsorbed anti-viral serum, there was marked fluorescence, indicating that this unadsorbed anti-viral serum contained antibody for the normal host component.

On the basis of these two results it was apparent that specific anti-viral serum which did not contain any antibody against the host component must be used. After repeated trials, this was easily achieved by adsorbing the serum with normal CAM powder as described in "Materials and Methods".

Fig. 1. Sedimented virus fixed on the slide and stained with unadsorbed anti-viral serum by the fluorescent spot technique. Notice marked fluorescent granules

Fig. 2. Sedimented virus fixed on the slide and stained with anti-NSP serum by the fluorescent spot technique. Notice moderately fluorescent area, showing fuzzy fluorescent granules of various sizes

Fig. 3. Aluminum phosphate gel purified virus, eluate-3 stained with anti-viral serum adsorbed with normal CAM by the fluorescent spot technique. Notice the marked fluorescent area, showing tiny particulate fluorescences arranged in a regular fashion

When a NSP antigen on slides was tested with this adsorbed anti-viral serum, no fluorescence appeared.

Sedimented viral specimens were tested with this factor serum in parallel with unadsorbed serum. Moderate fluorescence appeared with the former, whereas marked fluorescence was found with the latter. This difference was ascribed to the presence of antibody against host component in the unadsorbed serum.

Preparations of eluates -1, -2 and -3 were reacted with the adsorbed anti-viral sera in parallel with unadsorbed anti-viral serum. No difference was found between these two sera with any of these three antigens (Fig. 3). Thus these eluates probably did not contain appreciable amounts of the host components, which were detectable in the sedimented virus.

To prove this the above three eluate preparations were reacted with anti-NSP serum, using sedimented virus as a control. As in the second experiment mentioned above, moderate fluorescence was observed with sedimented virus. Whereas eluate-1 showed only slight fluorescence. Fluorescence with the eluates-2 and -3 was almost negligible.

These results are summarized in Table I. The fact that both eluates-2 and -3, which react with the factor anti-viral serum, did not react significantly with anti-NSP serum indicates that fairly large amounts of host components had been removed from these two fractions. The details of the serological analysis of these preparations by agar gel diffusion assay are reported in a following paper.⁷⁾ When eluates-2 and -3 were tested with anti-NSP serum, no precipitin boundaries

TABLE I. Degree of Fluorescence When Viral and NSP Antigens Were Tested with Anti-viral and Anti-NSP Rabbit Sera by the Fluorescent Spot Technique

Antigen	When tested with antibody prepared against		
	Virus		Normal allantoic fluid
	Unadsorbed	Adsorbed with normal-CAM	
Sedimented virus	##*	+	+
Aluminum phosphate gel purified virus	Eluate-1	##	+
	Eluate-2	##	±
	Eluate-3	##	±
Normal sedimentable component	##	—	##

* Degree of fluorescence: ## marked, + moderate, + slight, ± trace, and — no fluorescence.

of soluble and sedimentable proteins appeared. Electron-microscopic studies also supported the above conclusions.⁷⁾

2. Hemagglutination inhibition titration using purified virus

The results described above suggest that sedimentable protein, which is known to be difficult to remove from the surface of virus particles was eliminated by aluminum phosphate gel purification. If this is the case, when these purified viral antigens were tested carefully by the HAI test with adsorbed anti-Sendai virus rabbit serum, a lower titer of serum antibody than the titer obtained with crude viral antigen should be found, because of the increased viral antigenic surface of the purified particles. Table II shows the results obtained. Eluate-3 was twice

TABLE II. Antigenicity in Hemagglutination Titration and Hemolytic Activity of Various Viral Preparations

Antigen		HAI titer against anti-Sendai virus serum*	HA unit/ml	HL unit/ml	HL/HA†
Sedimented virus		$10^{-2.5}$	$10^{4.5}$	$10^{3.8}$	$10^{-0.7}$
Aluminum phosphate gel purified viral antigens	Eluate-1	$10^{-2.4}$	$10^{3.0}$	$10^{1.6}$	$10^{-1.4}$
	Eluate-2	$10^{-2.2}$	$10^{4.5}$	$10^{3.4}$	$10^{-1.1}$
	Eluate-3	$10^{-2.1}$	$10^{4.7}$	$10^{3.5}$	$10^{-1.2}$
	Eluate-4	$10^{-2.1}$	$10^{4.3}$	$10^{3.1}$	$10^{-1.2}$

* For the titration of HAI, adsorbed anti-Sendai virus rabbit serum was used.

† HL/HA indicates the ratio of hemolytic to hemagglutinating units.

as active as viral antigen when compared with the sedimented virus. The possibility of a lowering due to the aggregation of viral particles with these purified specimens was disproved by examining the fractions by electron microscopy.

3. Reduction of the hemolytic activity with purified virus

When the hemolytic activity of purified virus preparations was compared with that of the sedimented virus preparation, the results shown in Table II were obtained. The ratio of hemolytic to hemagglutinating units was calculated for each preparation and this ratio was used as a criterion of purity. There was a reduction in this ratio after aluminum phosphate gel purification.

DISCUSSION

The immunofluorescent technique developed by Coons was used in these experiments to detect viral particles fixed on a slide glass. Although the minimum number of viral particles required in the test to detect fluorescence was not studied, experiments made recently in this laboratory show that the particles of L cell-born Sendai virus adsorbed on the surface of an L cell could be counted.¹⁵⁾ This shows the sensitivity of the test and if the fixation of virus particles on the glass slides is carried out under optimal conditions, this spot technique should be equally sensitive.

In the test, 0.05 ml of a suspension containing 10,000 HA units/ml was spread in a circular area of 3 mm diameter on a slide. When this suspension was diluted 100 times there was still marked fluorescence. However, in testing the purity of a virus sample by this spot technique, it was found best to use a concentrated virus sample. Thus when a highly purified virus suspension was reacted with adsorbed anti-viral serum, tiny fluorescent particles arranged in a regular fashion were found all over the area, as if the flocculation reaction had occurred around each virus particle. Whereas, when the virus tested was obtained only by means of one-cycle differential centrifugation, fuzzy fluorescent granules of various sizes were seen. Their margins were indistinct and they were distributed irregularly over the area. From this difference, it was possible to judge the degree of purity of the sample. To recognize this difference in the spot test, a concentrated virus sample was more suitable than a dilute one.

Because of the recent tendency to add adjuvant in influenzal vaccines, the demand for viral products free of extraneous material is increasing. The simple and rapid test with anti-NSP serum^{16, 17)} described in this report will be very useful to test the purity of such preparations.

The hemagglutination inhibition titer was low in these experiments when purified virus was substituted for sedimented virus. The same effect was reported recently by Hausler and Dick¹⁸⁾ in experiments with virus extracted from a zinc-virus precipitate with glycine. The purification procedure used here may

remove the outer layer of the viral particles, exposing more antibody-binding sites. When the hemolytic activity of crude and purified preparations was compared, the reduction in hemolytic activity was found to be almost parallel to the decrease in the hemagglutination inhibition titer. The absence of hemolytic activity in methanol-purified egg-born virus,⁹⁾ small hemagglutinins detectable in the infected chorio-allantoic membrane, and L cell-born virus¹⁹⁾ has already been established in this laboratory.

The possible existence of host components as intrinsic entities on the surface of viral particles^{20, 21)} still remains as the critical point for further studies. Although our most purified preparation showed faint fluorescence, this was so weak as to be described as almost negligible in the text. The spot technique described here could also be applied to other purposes, i.e. titration of antibody against various viruses, identification of various viral antigens and testing the purity of various virus preparations.

SUMMARY

The fluorescent spot technique was developed so that the flocculation reaction between viral particles and antibodies could be seen quickly and simply on a glass slide. This technique was readily available for testing the purity of the Sendai virus during its purification. Immune serum prepared against normal sedimentable material of uninfected eggs was particularly useful to test for the presence of host components in purified viral suspensions. A highly purified fraction of high HA titer obtained by adsorption on, and elution from aluminum phosphate gel, was shown by this spot technique to be almost free from normal components. When such a purified preparation was used for HAI titration, the antibody titer was low because of the increased viral antigenic surface around the particles. The hemolytic properties known to be associated with Sendai virus were also reduced in such a purified preparation.

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