Improvements in the Passive Hemagglutination Technique for Serological Detection of Plant Viruses

R. Rajeshwari, D. V. R. Reddy, and N. Iizuka

First and second authors, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), ICRISAT Patancheru Post Office, 502324, A. P. India; third author, Hokkaido National Agriculture Experiment Station, Hitsujigaoka, Sapporo, Japan.
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ABSTRACT

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The use of some samples of normal rabbit serum for diluting antigen in passive hemagglutination (PHA) resulted in nonspecific agglutination. Bovine serum albumin at 0.5% concentration prevented such nonspecific agglutination. The sensitivity of sheep red blood cells (RBC) treated with glutaraldehyde before they were tanned and sensitized, was comparable to that of fresh RBC. Glutaraldehyde-treated cells could be preserved at 4–6 C for 2 mo without detectable loss in sensitivity. Better agglutination was obtained when sensitization of glutaraldehyde-treated cells with globulins was done at pH 7.0 rather than at 6.4 or 5.2. In comparative experiments, the sensitivity of PHA with purified peanut green mosaic virus was 40–80 times that of agar gel diffusion and precipitin ring tests, respectively. The minimum concentration of peanut green mosaic virus detected by PHA was 0.75 μg/ml.

The outstanding advantages of the passive hemagglutination (PHA) technique are its great sensitivity for the detection of plant viruses (1,8,10,12) and its ease of operation without need for specialized equipment or reagents. The test can be used to detect viral antigen in crude plant extracts prepared in phosphate-buffered saline containing normal rabbit serum (NRS) (1,6,8,9,). We found that some samples of NRS even after heat inactivation (4) resulted in nonspecific agglutination. This paper describes an alternative method to avoid nonspecific agglutination.

It is advantageous to have a stable preparation of sensitized red blood cells (RBC) since fresh RBC lyse within four days. Formalin treatment of RBC has been used for preservation (5), but slightly lower titers were obtained (1). The sensitivity of glutaraldehyde (GA)-treated RBC (3) was similar to that of fresh RBC for the detection of antibody of *Entamoeba histolytica* (7). In this paper we describe the application of GA-treated RBC in PHA for the detection of plant viral antigens without detectable loss in sensitivity.

MATERIALS AND METHODS

Sources and preparation of viruses. Peanut green mosaic virus (PGMV), a potyvirus infecting peanut in India (G. Sreenivasulu et al, *unpublished*), and tomato spotted wilt virus (TSWV) isolated from a single local lesion produced on French bean and cowpea, respectively, were maintained on groundnut by periodic sap inoculations.

PGMV was purified by organic solvent clarification, concentration in a sucrose cushion containing polyethylene glycol, and rate-zonal and quasi-equilibrium zonal density gradient centrifugation in sucrose solutions. Polyacrylamide gel electrophoresis of polypeptides and electron microscopy revealed no detectable impurities in the purified virus preparation (G. Sreenivasulu et al, *unpublished*). Virus concentration was measured spectrophotometrically and the extinction coefficient ($E^{0.1\%}_{260nm}$) of the virus ($A_{260}/A_{280} = 1.20$) was assumed to be 3.0.

Preparation of antisera and globulins. Antiserum for TSWV was supplied by G. V. Gooding, Jr., University of North Carolina, Raleigh. Antiserum for PGMV was prepared by injecting rabbits intramuscularly with virus preparations emulsified with an equal volume of Freund's incomplete adjuvant. Four intramuscular injections (each 1–2 mg of virus) were given at weekly intervals and the rabbits were bled 4 wk after the last injection. The titer of the antiserum, as determined by the precipitin ring test, was 1/640.

Serum globulins were prepared by ammonium sulfate precipitation. One part of antiserum was diluted with nine parts of half-strength PBS (PBS = 0.15 M phosphate buffer, pH 7.2, mixed with equal volumes of 0.85% NaCl). An equal volume of saturated $(NH_4)_2SO_4$ solution (pH adjusted to 7.8 with 2N NaOH) was added drop by drop with stirring at low temperature. The mixture was stirred for 2–3 hr at 4 C. The precipitate was collected by centrifugation for 20 min at 2,000 *g* and resuspended in 0.85% NaCl to the original volume of the serum. The serum globulins were reprecipitated in ammonium sulfate and the γ-globulins were dissolved in 0.85% NaCl to yield five times the original volume of the serum. Ammonium sulfate was removed by dialysis against 0.85% NaCl at 4 C for 1 day (four changes of 1 L each). All dilutions of globulins employed for coating the RBC refer to the original volume of the antiserum.

Passive hemagglutination test. Sheep RBC were used in all tests. Blood was collected aseptically either in 1.2 volumes of Alsever's solution (4) or in the presence of 0.1% heparin (Biological Evans, India) and stored for 1 day at 4 C. The cells were washed with PBS by centrifugation at 1,200 rpm for 3 min. After three washings, 1.0 ml of packed cells was resuspended in 15.7 ml of PBS (6% suspension of RBC) and treated with an equal volume of freshly prepared 0.01% tannic acid (Fischer Chemicals, A-310), in PBS (final concentration of tannic acid was 0.005%). Tannic acid, when used at a final concentration of 0.01% and 0.025%, occasionally resulted in nonspecific agglutination. RBC were tanned by incubating in a refrigerator for 20 min with intermittent mixing. Excess tannic acid was removed by washing twice with PBS and resuspending the cells in PBS to give a 3.0% RBC suspension. The cells were sensitized with γ-globulin at 37 C for 30 min. Excess globulin was removed by two washings in PBS and sensitized cells were resuspended to give a 3.0% suspension in a diluent employed for preparing the antigen. The test was conducted in Lucite plates containing wells of 1.0 ml capacity. Test antigens (0.5 ml) and antibody-sensitized cell suspension (0.08 ml) were added to each well. The plate was shaken gently until a uniform RBC suspension was observed and then it was left at room temperature for about 2 hr before being placed in a

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refrigerator. Results were recorded after overnight incubation.

Dilutions of crude plant extracts were based on the original weight of leaf material. Extracts were prepared by triturating in nine times their weight of 0.05 M phosphate buffer, pH 7.0, containing 0.02 M 2-mercaptoethanol. The sap was filtered through cheesecloth and clarified at 5,000 rpm (2,500 *g*) for 10 min in a Remi K-24 refrigerated centrifuge. Tests were made on serial twofold dilutions of supernatants.

The following controls were included for each testing: extracts from healthy and infected leaves plus unsensitized RBC, extracts from healthy leaves plus globulin-sensitized cells, extracts from healthy and infected leaves plus cells sensitized with globulins of a heterologous antiserum, diluent employed for preparing antigens plus sensitized cells, and diluent employed for preparing antigens plus unsensitized tannic acid treated cells.

In the case of a positive reaction, RBCs formed a smooth mat, frequently with a serrated margin, on the bottom of the well. A negative reaction consisted of a discrete red ring at the periphery of the well.

Treatment of cells with glutaraldehyde. Red cells were treated with GA, as described by Krupp (7) with slight modifications. Fresh RBC, washed twice in 0.85% saline and three times in PBS, were chilled at 4 C for about 30 min. Packed cells were diluted to a 3% suspension with cold 1% GA solution (50% glutaraldehyde (BDH Chemicals, England) or 25% glutaraldehyde (Sigma Grade 1), prepared in PBS, and incubated at 4 C for 30 min with occasional mixing. Excess GA was removed by washing twice with PBS and five times with distilled water. Packed cells were resuspended to give 30% concentration in PBS containing 0.5% BSA and 0.05% sodium azide and stored at 4–6 C. Stored RBC were washed three times in PBS before they were used for tanning.

Precipitin tests. The precipitin ring interface test was done as described by Reddy et al (8). Double diffusion was done by using a gel consisting of 0.8% Ionagar in PBS containing 0.05% sodium azide and 0.5% 3,5,-diidosalicylic acid (Sigma); presumably the latter helps in the diffusion of long virus particles by breaking them into shorter segments.

RESULTS AND DISCUSSION

Prevention of nonspecific agglutination of antibody coated cells. The optimum dilution of globulins from antisera to TSWV and PGMV for sensitizing tanned RBC were 1:2,000 and 1:5,000, respectively.

NRS has been used to prevent nonspecific agglutination of sensitized RBC (1,4,8). When NRS, from 10 different rabbits was heat treated at 56 C for 30 min and used at 1% of 0.05% concentration in PBS, as a diluent to prepare antigens, three NRS samples consistently caused nonspecific agglutination of peanut leaf extracts. This effect of NRS also was observed when leaf extracts from *Lycopersicon esculentum* Mill., *Phaseolus vulgaris* L. (Prince and Topcrop), *Pisum sativum* L., and *Vigna unguiculata* (L.) Walp. Subsp. *unguiculata* were used. In preliminary experiments, normal and inactivated fetal bovine serum (FBS) (V. P. Chest Institute, India) casein (Technical grade, Sigma), bovine serum albumin (BSAM Sigma A-4398) at 0.1, 0.2, 0.5, and 1.0% concentrations, prepared in PBS, and PBS alone were tried in the place of NRS to prevent nonspecific agglutination. At all concentrations of casein and FBS employed, and in PBS without any supplement, nonspecific agglutination was observed. BSA at 0.5 and 1% concentration prevented nonspecific agglutination. In two experiments with PGMV and TSWV, similar antigen titers were obtained whether the antigens were prepared in 0.5% BSA or 1% NRS; samples of NRS that did not give nonspecific agglutination were employed in these tests. In all subsequent experiments, PBS containing 0.5% BSA without NRS, was used to prepare antigen dilutions.

Preservation of RBC. The sensitivity of GA-treated cells, some of which were stored for 2 mo, was comparable to that of fresh RBC for the detection of TSWV and PGMV antigens (Table 1). Cells stored beyond 2 mo were not suitable for sensitization. The following experiments were performed on GA-treated cells stored for less than 2 mo.

Effect of pH on sensitization of GA-treated tanned cells. GA-treated tanned cells, at 3.0% concentration, were suspended in PBS of pH 5.2, 6.4 and 7.0. Cells were sensitized with anti-TSWV and anti-PGMV globulins in 0.85% NaCl and then washed and resuspended in PBS, pH 7.2. In two experiments on each antigen, titers obtained as a result of sensitization at different pH values were similar. However, cells that had been coated at pH 7.0 (Fig. 1) resulted in better agglutination in the lower concentrations of the antigens and the titers were easier to compute. Agglutination patterns obtained for cells coated at pH 5.2 were comparatively more difficult to read.

Order of adding the reagents for sensitization of GA-treated tanned cells. The effect of the sequence of adding the various reagents to sensitize the cells (one volume of tanned RBC to one volume of globulins mixed with four volumes of PBS (1,2,11), or one volume of globulins to nine volumes of tanned RBC as used in the present work) was compared using globulins from TSWV antiserum. The final cell concentration (2.7%), globulin dilution (1/2,000) and the pH (7.0) employed for sensitization were identical for both procedures. In three experiments, both methods of sensitization gave identical antigen end points of 1/12,800.

Relative sensitivity of PHA, precipitin ring interface, and agar gel diffusion. GA-treated tanned cells (3%) were coated with

TABLE 1. Comparison of the sensitivities of glutaraldehyde-treated and fresh red blood cells in passive hemagglutination with two peanut viruses

Virus	Exp. no.	Reciprocal of PHA titers[a]	
		Fresh cells	Glutaraldehyde-treated cells
Tomato spotted wilt[b]	1	12,800	12,800
	2	25,600	25,600
	3	20,480	20,480[c]
Peanut green mosaic	4	6,400	6,400
	5	6,400	6,400[c]

[a] Dilutions for sensitization of globulins from tomato spotted wilt virus and peanut green mosaic virus antisera were at 1/2,000 and 1/5,000, respectively.

[b] Dilutions were prepared in PBS containing 0.5% BSA and were based on original weight of leaves.

[c] Glutaraldehyde-treated cells stored at 4–6 C for 8 wk were used for sensitization.

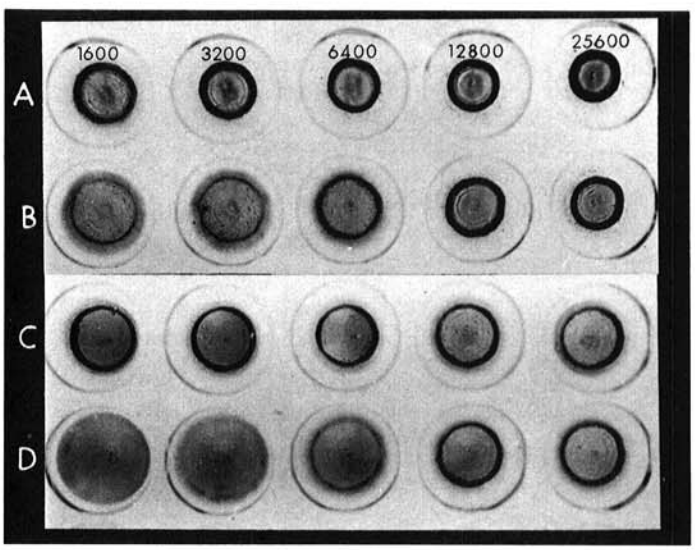

Fig. 1. Effect of pH on sensitization of glutaraldehyde-treated tanned cells with globulins from peanut green mosaic virus antiserum. Each horizontal row contains serial two-fold dilutions of peanut leaf extracts starting from the left, at 1/1,600 to 1/25,600 dilution. **A,** Red blood cells coated at pH 6.4 and tested with healthy peanut leaf extracts and **B,** Extracts from peanut green mosaic virus-infected leaves. **C,** Red blood cells coated at pH 7.0 and tested with healthy peanut leaf extracts and **D,** extracts from peanut green mosaic virus-infected leaves.

globulins from PGMV antiserum at a final dilution of 1/5,000 at pH 7.0. Dilutions of purified preparations of PGMV were prepared in PBS containing 0.5% BSA. The purified virus preparation also was titrated by the precipitin ring interface test (8) and Ouchterlony's agar gel diffusion test. In tests with precipitin ring interface and agar gel diffusion, the maximum virus titers were obtained with globulin dilutions of 1/40 and 1/4, respectively. The minimum concentration of virus detected by the PHA test was 0.75μg/ml compared to minimum concentrations of 31.25 and 62.5 μg/ml, respectively, detected by precipitin ring interface and agar gel diffusion tests. The PHA test was thus 40–80 times more sensitive than were the other two tests.

Our results have confirmed previous reports (1,8,10,12) that the PHA test is a highly sensitive serological method. GA-treated cells could be stored for 2 mo at 4–6 C without detectable loss in sensitivity for detecting two different plant viral antigens. Previous studies with formalinized cells indicated that the cells could be stored up to 8 wk at −14 C (1). Because of large variation in freezer temperature we were unable to perform experiments on the effect of freezing. Experiments are underway to determine if lyophilizied cells can be used without loss in sensitivity.

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