The Detection of Low Concentrations of Double-Stranded Ribonucleic Acid with Iodine-125 Labeled Antiserum

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ABSTRACT

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Antiserum prepared against double-stranded (ds) RNA poly I·poly C reacted in Ouchterlony double-diffusion tests with poly I·poly C (titer = 1:16) and poly A·poly U (titer = 1:8), but not with yeast RNA or calf thymus DNA. The antiserum also removed the poly I·poly C zone from centrifuged sucrose density gradients. When the antiserum against poly I·poly C was labeled with ^{125}I and added to varying concentrations of poly I·poly C, it detected as little as 10 ng in 0.01 ml of buffer. This labeled antiserum also reacted with similar concentrations of pea enation mosaic virus (PEMV) replicative form (RF) RNA, ϕ6 RNA, and poly A·poly U. Labeled normal serum gave only background counts when tested with any of these RNA's. In addition, the labeled antiserum against poly I·poly C reacted with dsRNA in nucleic acids extracted from nuclei or vesicles of PEMV-infected cells, but not with nucleic acids extracted from chloroplasts of PEMV-infected cells or with nucleic acids extracted from any healthy cell fraction.

Studies of the replication of single-stranded (ss) RNA plant viruses, have shown that double-stranded (ds) RNA [called replicative form (RF)] and partially double-stranded RNA [called replicative intermediate (RI)] are produced during virus replication. One or both of these RNAs are believed to be intermediates in virus replication (3, 10). The replication of pea enation mosaic virus (PEMV) has been studied intensively in both intact plants (3) and in isolated, artificially infected pea nuclei (8). In both cases, PEMV specific dsRNA was isolated.

Antibodies against dsRNA were prepared first by Schwartz and Stollar (9). The technique was employed by de Zoeten et al. (2) who used ferritin-labeled antibody to obtain evidence that the nucleus is the site of PEMV replication. However, antibodies against dsRNA have not been used extensively in virology because they do not allow detection of low concentrations of dsRNA.

Labeling antibodies with ^{125}I is a technique which frequently is used to enhance detection of low concentrations of antigen. This technique was first used in plant virology by Langenberg (6). We labeled antibodies against dsRNA with ^{125}I to increase our ability to detect ds forms of RNA. This paper reports the preparation and labeling of antibodies against poly I·poly C and their subsequent use in detecting PEMV replication products.

MATERIALS AND METHODS

Preparation of antiserum.—Antiserum against dsRNA was prepared by a procedure similar to that used by Schwartz and Strollar (9). Two-hundred and fifty μg of synthetic poly I·poly C (minimum MW 100,000) per ml 2× SSC (standard saline citrate; 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) were mixed with an equal volume and concentration of methylated bovine serum albumin (MBSA). One-half ml of this solution was emulsified with 0.5 ml of Freund's complete adjuvant and injected intramuscularly into the thighs of young New Zealand white rabbits (2), which previously had been bled for normal serum. Intramuscular injections were repeated the next 2 wk. The third week, each rabbit received a 1.0-ml intravenous injection of 125 μg poly I·poly C in SSC without adjuvant. One wk later (3 wk after the initial injection) the rabbits were exsanguinated, and the serum was collected, titered, and stored frozen in 1-ml aliquots. This serum will be referred to as dsRNA As.

Ouchterlony agar double-diffusion tests. An agar solution that consisted of 0.60% Ionagar No. 2, 0.80% NaCl, 0.09 M glycine, pH 8.0, and 0.009 M sodium azide was autoclaved and poured into 6-cm diameter plastic petri dishes. Wells were cut 1 cm apart on center with a No. 1 cork borer, to form the center and corners of a regular hexagon. The wells were charged with 0.01 ml of the appropriate antiserum or antigen, and the results were read 24 hr later.

Density gradient centrifugation serology. Density gradient centrifugation (DGC) serology was performed as described by Ball and Brakke (1). The continuous gradients consisted of 75 to 300 mg sucrose/ml SSC. The centrifugation conditions for analyzing the antigen-antibody reactions were 45,000 rpm for 5 hr in an SW 56 rotor. The prolonged high-force centrifugation was

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necessary to sediment the poly I·poly C through the gradients.

Labeling antibodies against dsRNA.—The DEAE cellulose-purified immunoglobulin (IG) was dialyzed against 0.05 M sodium phosphate, pH 7.5, and concentrated with ammonium sulphate (0.22 g/ml IG solution). The IG was dissolved in 0.05 M sodium phosphate, pH 7.5, and centrifuged at 24,000 rpm for 24 hr on 75 to 300 mg sucrose/ml 0.05 M sodium phosphate, pH 7.5, density gradients in an SW27 rotor at 5 C. The IG zone (1.5 cm from the meniscus) was collected, dialyzed against the above phosphate buffer, and concentrated by ammonium sulphate precipitation.

The IG was iodinated by the procedure of Greenwood et al. (4). One mg of IG in 0.6 ml of sodium phosphate buffer, pH 7.5, was placed in a cold scintillation vial to which 1 mCi ^{125}I (specific activity 17.3 Ci/mg) and 0.1 ml fresh 200 μg chloramine-T/ml were added. After being shaken for 10 min, the reaction was terminated by adding 0.14 ml of freshly made 200 μg sodium metabisulfite/ml. Then the labeled antibodies were separated from the unreacted iodine with a BIO-RAD polypropylene Econo column containing BIO-RAD AG1-X4 anion exchange resin which previously had been equilibrated with 0.05 M sodium phosphate buffer, pH 7.5, and washed with 2% BSA to help eliminate nonspecific binding. The antibody solution, which eluted from the column in 1 ml, was dialyzed overnight against 1 liter of water, filtered with a Millipore filter (Selectron BA 85), and used within 1 wk.

Radioimmunoassay.—A mixture of 5 μliters of purified ^{125}I-labeled dsRNA antibodies (5 μg) and 5 μliters (5 μg nucleic acid) of antigen in 2× SSC was incubated for 1 hr at room temperature before storage overnight at 4 C. The next day the 0.01-ml samples were suspended in 5 ml of SSC and the precipitates were collected on Selectron BA 85 filters by filtration with a Millipore apparatus. The filters were washed four times with 5 ml SSC and twice with 80% (v/v) ethanol. They then were placed in scintillation vials, suspended in modified Bray's scintillant (5), and counted in a Nuclear-Chicago Mark II liquid scintillation spectrometer (^{3}H window).

RESULTS

Reactions of nonlabeled antiserum against dsRNA.—Crude antiserum (dsRNA As), which had been collected from each of two rabbits that had been immunized with MBSA-poly I·poly C, was titered by reaction with 1 mg poly I·poly C/ml SSC in Ouchterlony agar double diffusion plates. The serum from one rabbit had a titer of 1:16, the other, 1:8. The serum with 1:16 titer was used in all subsequent experiments.

Two tests were used to determine the specificity of the antiserum (As). First, Ouchterlony double diffusion was used to establish that the dsRNA As reacted strongly with poly I·poly C and to a lesser extent with poly A·poly U. Normal serum did not react with either poly I·poly C or poly A·poly U in these tests. The dsRNA As also did not react with either calf thymus DNA or yeast RNA.

A serological test employing sucrose density gradients was used to insure that the precipitate observed in the Ouchterlony plates was due to precipitation of poly I·poly C by the dsRNA As. Figure 1 shows that the nondiluted antiserum against poly I·poly C removed the poly I·poly C zone (Fig. 1-C) from sucrose density gradients, and that normal serum had little effect on this zone (Fig. 1-E). This confirmed that the dsRNA As could precipitate poly I·poly C.

Fig. 1 (A to E). Ultraviolet absorption (254 nm) profile of centrifuged sucrose gradients. The gradients were continuous 75-300 mg sucrose/ml standard saline citrate (SSC), centrifuged at 45,000 rpm for 5 hr in an SW 56 rotor. The material layered on the gradients was 0.2 ml of **A)** poly I·poly C, **B)** As against poly I·poly C, **C)** nondiluted As against poly I· + poly I·poly C, **D)** 1:10 dilution of As against poly I·poly C + poly I·poly C, **E)** nondiluted normal serum + poly I·poly C. The above dilutions of As were mixed 1:1 (v/v) with 25 μg/ml antigen in 2× SSC so that the final antigen concentration was 12.5 μg/ml SSC.

Reactions of ^{125}I-labeled antibodies against dsRNA.—Since As prepared against poly I·poly C should react with any dsRNA but not substantially with DNA or ssRNA (8), it was hoped that it could be used as an analytical tool to detect ssRNA virus replication. However, the titer of the serum was too low to allow visual detection of low concentrations of virus RF. Therefore, the antibody molecules were labeled in vitro with ^{125}I so that small amounts of precipitate could be detected.

Purified, labeled antibodies (Ab) with a specific activity of approximately 200,000 cpm/μg were obtained. However, this activity proved to be too high, resulting in high background counts due to nonspecific binding to the Millipore filters. Therefore, the labeled Ab suspensions were diluted 1:10 with water to achieve acceptable background levels.

The labeled Ab first were used to prepare a standard curve to estimate the effect of antigen (Ag) concentration on precipitable counts. Varying concentrations of poly I·poly C were mixed with labeled Ab (specific activity, approximately 20,000 cpm/μg), and the precipitate was collected on Millipore filters and counted. The results (Fig. 2) show a linear relationship between Ag concentration and precipitable counts in the range tested. By means of this labeled Ab we consistently could detect poly I·poly C concentrations as low as 1 μg/ml which is equal to 10 ng because the sample volume was 0.01 ml. Replicates within the same experiment usually did not vary much (maximum 25%). When an experiment was repeated several days later, the numbers could vary as much as 300%, although the trend was the same. This decrease in cpm with time probably was due to denaturation of the labeled Ab.

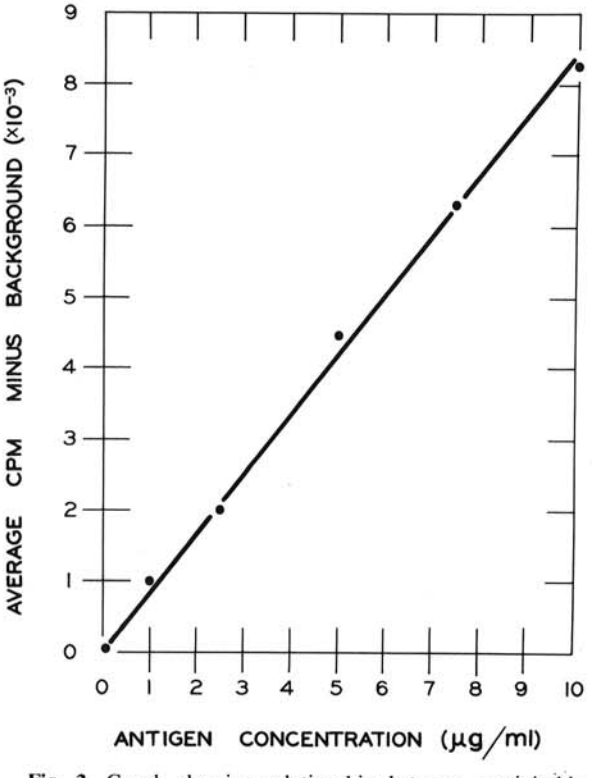

Fig. 2. Graph showing relationship between precipitable counts and poly I·poly C concentration. The varying concentrations of antigen were mixed with a 1:10 dilution of ^{125}I-labeled antibody (Ab) against poly I·poly C (specific activity approximately 20,000 cpm/μg). The precipitate was collected on Millipore filters and counted. The antigen concentrations are the final concentrations after mixing with Ab. The reaction buffer was standard saline citrate. Background was 411 cpm.

Once it had been established that is was feasible to use ^{125}I-labeled antibodies against poly I·poly C to detect small amounts of poly I·poly C, it was necessary to determine if these same antibodies would detect low levels of other dsRNAs. Therefore, various nucleic acids were mixed with the ^{125}I-labeled dsRNA Ab, and any precipitate was collected on Millipore filters and counted. The results (Table 1) show that the labeled dsRNA detected small amounts of all dsRNA's tested (poly I·poly C, poly A·poly U, ϕ6 RNA, PEMV RF), but did not react appreciably with dsDNA (calf thymus DNA) or ssRNA (yeast RNA). Labeled normal serum did not react with dsRNA. It was interesting that the dsRNA Ab showed some specificity among dsRNAs. It reacted much better with the RNA used for immunization (poly I·poly C) than with the other dsRNAs.

Since ^{125}I-labeled dsRNA Ab detected PEMV RF, the critical experiment was to determine if it could be used as an analytical tool to detect viral dsRNA in the presence of host nucleic acids. Pea enation mosaic virus in peas was the obvious system to study because it was readily available, the mechanism of PEMV replication had been studied (3, 8), and the intracellular site of PEMV replication had been established in the past (2, 8).

Fractions from infected and healthy peas (8) and healthy pea nuclei which had been incubated with PEMV-RNA for 16 hr at 30 C were homogenized and phenol-extracted to obtain their total nucleic acid (8). This nucleic acid was mixed with labeled dsRNA Ab, and the precipitate was collected on Millipore filters (Selectron BA 85) and counted. Average counts and calculated percent dsRNA are presented in Table 2. The

TABLE 1. Reaction of ^{125}I-labeled antibodies with various nucleic acids

Serum[a]	Antigen[b]	Average cpm[c]
poly I·poly C As	poly I·poly C	8,681
poly I·poly C As	poly A·poly U	4,869
poly I·poly C As	ϕ6 RNA	2,435
poly I·poly C As	PEMV RF	1,509
poly I·poly C As	calf thymus DNA	658
poly I·poly C As	yeast RNA	361
poly I·poly C As	buffer	411
normal serum	poly I·poly C	316
normal serum	poly A·poly U	331
normal serum	buffer	448

[a]Antibodies were diluted to a specific activity of approximately 20,000 cpm/μg.

[b]Antigen concentration was 10 μg/ml in 0.01 ml of standard saline citrate.

[c]Counts in the precipitate collected on Millipore (Selectron BA 85) filters.

calculated percent dsRNA represents the percent of total nucleic acid calculated to be dsRNA by subtracting background counts (buffer), determining the concentration of dsRNA using PEMV RF value in Table 1, and dividing this concentration by the concentration of total nucleic acid (determined spectrophotometrically using $\epsilon_{260} = 25$). For example, vesicles from infected peas gave 19,946 cpm. This equalled 18,700 after subtracting the 1,246 buffer value. Ten μg PEMV RF/ml gave 1,509 - 448 or 1,061 cpm. Therefore, the concentration of dsRNA in the nucleic acid extracted from vesicles from PEMV-infected peas was $18{,}700/1{,}061 \times 10 = 176$ μg/ml. The concentration of total nucleic acid was 1 mg/ml, making the calculated percentage of dsRNA 17.6%. In the quantitation of these experiments the assumption has been made that linearity in the Ab-Ag relationship holds for higher concentrations of the heterologously as well as homologously tested dsRNAs.

The conclusion that can be drawn from the data in Table 2 is that by means of ^{125}I-labeled antibodies against dsRNA, viral dsRNA, and thus viral replication, can be detected in the presence of an excess of host nucleic acids. Pea enation mosaic virus replication intermediates were present in vesicles and nuclei from infected cells, but not in chloroplasts from infected cells or chloroplasts or nuclei from healthy cells. Table 2 also shows that healthy pea nuclei primed with PEMV-RNA could synthesize dsRNA. These results are consistent with previously reported conclusions (2, 7, 8).

DISCUSSION

This paper describes a technique for detecting low levels of viral dsRNA. ^{125}I-labeled antibodies against dsRNA successfully detected minute amounts of PEMV RF RNA in the presence of an excess of host DNA. Theoretically, this procedure should be applicable to many experiments which involve the detection of low levels of ssRNA virus replication products. This includes problems such as localization of virus replication sites, indexing plants for latent viruses, or studying virus replication in vectors.

Table 2 represents an attempt to measure the amount of dsRNA present in a given sample. Similar samples previously have been analyzed for their content of dsRNA by molecular hybridization (7, 8). The amounts of dsRNA from various sources as determined by the two methods compare favorably except for the nuclei sources in which dsRNA concentration determined by the dsRNA Ab technique was two to four times higher than the dsRNA concentration determined by molecular hybridization. The reason for this discrepancy is unknown, but there are at least two possible explanations. First, the presence of relatively high concentrations of DNA may alter the solution so as to increase dsRNA Ab binding or the precipitation of the dsRNA Ab complex. Second, infected, but not healthy, nuclei might contain dsRNA which is not virus-specific; this would be detected by dsRNA Ab, but not by hybridization. An alternate explanation for the discrepancy in the concentration of dsRNA in infected nuclei is that the hybridization procedure underestimated the dsRNA concentration; controls, however, indicate this was unlikely. At any rate, although the labeled dsRNA Ab technique is useful for qualitative detection, it needs further refinement before it can be used as a quantitative tool.

The usual method for detecting ssRNA replication is by hybridization using a labeled ssRNA probe. This procedure has two distinct disadvantages. First, it is laborious, particularly if many samples need to be analyzed. Second, hybridization requires a ssRNA

TABLE 2. Determination of dsRNA in cellular fractions of healthy and pea enation mosaic virus (PEMV)-infected pea plants by means of ^{125}I -labeled antibodies[a]

Source of nucleic acid[b]	Average cpm[c]	Calculated % dsRNA[d]
Vesicles from PEMV-infected peas	19,946	17.6
Chloroplasts from PEMV-infected peas	1,880	0.6
Nuclei from PEMV-infected peas	9,001	7.3
Chloroplasts from healthy peas	1,408	0.2
Nuclei from healthy peas	1,878	0.6
Nuclei from healthy peas[e] primed with PEMV-RNA	15,004	13.0
Nuclei from healthy peas primed with PEMV-RNA. The extracted nucleic acid was treated with RNase (low salt)[f]	1,896	0.6
Buffer	1,246	...

[a]Antibodies against dsRNA were diluted to a specific activity of approximately 20,000 cpm/μg and reacted with the nucleic acid from various sources.

[b]Nucleic acid was prepared from the various components by phenol extraction and ethanol precipitation. For details, see Powell et al., Virology 78:135-143.

[c]Counts in the precipitate collected on Millipore (Selectron BA 85) filters.

[d]The calculated % dsRNA represents the ratio of the dsRNA concentration, estimated using the PEMV RF value (Table 1) as a standard, and the total nucleic acid concentration, estimated spectrophotometrically.

[e]Healthy pea nuclei were incubated with PEMV-RNA for 16 hr at 30 C. For details, see Powell and de Zoeten, Proc. Nat. Acad. Sci., USA 74:2919-2922.

[f]Ribonuclease (RNase) treatment consisted of incubation at 30 C for 40 min in 200 μg pancreatic RNase A/ml 0.1× standard saline citrate (SSC) and 300 units T_1 RNase/ml 0.1× SSC.

probe which is difficult to obtain for viruses which occur in low concentration or are difficult to purify. The labeled dsRNA Ab procedure does not have these disadvantages. Once the antiserum has been prepared, it takes comparatively little time or work to label it and use it to analyze many samples.

The labeled dsRNA Ab technique is not intended as a substitute for hybridization. It is intended as a procedure for screening samples for dsRNA as a prerequisite to hybridization. It is also intended as an independent technique to confirm conclusions based on hybridization.

LITERATURE CITED

1. BALL, E. M., and M. K. BRAKKE. 1969. Analysis of antigen-antibody reactions of two plant viruses by density-gradient centrifugation and electron microscopy. Virology 39:746-758.
2. DE ZOETEN, G. A., C. A. POWELL, G. GAARD, and T. L. GERMAN. 1976. In situ localization of pea enation mosaic virus double-stranded ribonucleic acid. Virology 70:459-469.
3. GERMAN, T. L., and G. A. DE ZOETEN. 1975. Purification and properties of the replicative forms and replicative intermediates of pea enation mosaic virus. Virology 66:172-184.
4. GREENWOOD, F. C., W. M. HUNTER, and J. S. GLOVER. 1963. The preparation of ^{131}I-labeled human growth hormone of high specific radioactivity. Biochem. J. 89:114-123.
5. KEMP, J. D., and D. W. SUTTON. 1971. Protein metabolism in cultured plant tissues. Calculation of an absolute rate of protein synthesis, accumulation, and degredation in tobacco callus in vivo. Biochemistry 10:81-88.
6. LANGENBERG, W. G. 1967. Radioactively labeled antibodies as a probe for the localization of tobacco mosaic virus protein in plant cells. Ph.D. Thesis, Univ. of California, Berkely. 125 p.
7. POWELL, C. A., G. A. DE ZOETEN, and G. GAARD. 1977. The localization of pea enation mosaic virus-induced RNA-dependent RNA polymerase in infected peas. Virology 78:135-143.
8. POWELL, C. A., and G. A. DE ZOETEN. 1977. Replication of pea enation mosaic virus ribonucleic acid in isolated pea nuclei. Proc. Nat. Acad. Sci., USA. 74:2919-2922.
9. SCHWARTZ, E. F., and B. D. STOLLAR. 1969. Antibodies to polyadenylate-polyuridylate copolymers as reagents for double-strand RNA and DNA-RNA hybrid complexes. Biochem. Biophys. Res. Commun. 35:115-120.
10. THACH, S. S., D. DOBBERTIN, C. LAWRENCE, F. GOLINI, and R. E. THACH. 1974. The mechanism of viral replication. Structure of replication complexes of encephalomyocarditis virus. Proc. Nat. Acad. Sci., USA. 71:2549-2553.