Immunogenicity of Rice Dwarf Virus-Ribonucleic Acid

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KITAGAWA, Y., MATSUMOTO, T., OKUHARA, E. and SHIKATA, E. *Immunogenicity of Rice Dwarf Virus-Ribonucleic Acid*. Tohoku J. exp. Med., 1977, 122 (4), 337-343 — Using rice dwarf virus (RDV)-RNA which was extracted from RDV and further purified by MAK-column chromatography, anti-RDV-RNA antibodies were produced in rabbits immunized with RDV-RNA-methylated bovine serum albumin complexes. The antisera, as analyzed by complement fixation, cross-reacted with synthetic double stranded RNAs (poly (A)·poly (U), poly (I)·poly (C)), but not with native or denatured DNA, rRNA, tRNA, 5 S RNA and nucleic acids from rice plants. RDV-RNA treated with heat or dimethylsulfoxide was markedly reduced in reactivity to the antisera. When RDV-RNA was digested with RNase A at low salt concentration, its complement fixation activity was abolished. In double diffusion tests, two different precipitation lines were demonstrated between the antiserum and RDV-RNA. One of the precipitation lines connected with those was formed between the antisera, poly (A)·poly (U) and poly (I)·poly (C). As regards immunoglobulin classes of the antibodies, one of the two rabbits employed had antibody activity only in IgM and the other, exclusively in IgG. — RDV-RNA; immunogenicity

Antibodies to synthetic double-stranded RNA have been elicited in rabbits by Schwartz and Stollar (1969). The antibodies produced reacted specifically with double-helical polynucleotides, and the antisera were used as a tool to detect various double-stranded RNAs (Moffitt and Lister 1973; Monckton and Naora 1974). Recently, Ikegami and Franki (1973) reported the presence of poly (I)·poly (C)-reactive antibodies in rabbit antisera for rice dwarf and maize rough dwarf virus particles, showing the presence of the antibodies to double-stranded RNA in the virus. In the meantime, Cunningham and Naysmith (1975) reported that fungal double-stranded RNA was immunogenic in a wide variety of mammalian species. When the fungal double-stranded RNA was injected intravenously in native form into mammals, high levels of antibodies were consistently obtained. Furthermore, in rabbit and

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rat antisera, antigen binding activity was not inhibited by synthetic polyribonucleotides, suggesting that these species can recognize additional antigenic structures, possibly particular sequences of bases, present in double-stranded RNA preparations.

In this laboratory, we have been studying the correlations between the antigenicity and antigenic structure of salmon sperm DNA. Interestingly, rice dwarf virus (RDV) contains double-stranded RNA and the chemical and physicochemical properties of this RNA have been characterized by Miura et al. (1966) and Sato et al. (1966). Considering this, we have attempted to elicit specific antibodies to RDV-RNA, hoping that an investigation of the antigenicity of this RNA, in parallel with the DNA research being done in our laboratory, would provide significant information. In this paper, we describe the production and characterization of antibodies to RDV-RNA. A preliminary report of these studies has recently appeared (Kitagawa and Okuhara 1975 a).

MATERIALS AND METHODS

Preparation of RDV-RNA

RDV particles from the infected rice plants were purified by a modification of the methods reported by Kimura (1973), and Kodama and Suzuki (1973). RDV-RNA was extracted from purified RDV particles by the phenol method (Miura et al. 1966) and further purified by column chromatography on methylated albumin Kieselguhr (MAK) (Kodama et al. 1968). Samples were loaded onto the column (2.5×24 cm) and eluted with linear salt gradients (0.4 to 1.6 M NaCl in 0.05 M phosphate buffer, pH 6.7) in a total volume of 800 ml. 10 ml fractions were collected at a flow rate of 40 ml/hr. The main peak fractions were collected, concentrated and treated further with Pronase E according to the method described by Stern and Littauer (1971). These procedures yielded immunochemically pure RDV-RNA. DNA, rRNA and tRNA of rice plants were separated and purified in the same way.

Immunization

The phenol extracted RDV-RNA was used for immunization. 125-250 µg of RDV-RNA in 0.9 ml of 0.15 M NaCl and an equal weight of methylated bovine serum albumin (MBSA) in 0.1 ml of water were mixed. The complex emulsified with an equal volume of complete Freund's adjuvant was injected intramuscularly into rabbits at one week intervals, and blood was drawn one week after the fourth injection. Complements present in the sera were inactivated by incubation at 56°C for 30 min and then cold hemagglutinins were removed by absorption on erythrocytes at low temperature. The sera thus prepared were lyophilized and kept at -20°C.

Dimethylsulfoxide (DMSO) treatment and cellulose column chromatography

RDV-RNA dissolved in 0.01 M NaCl-ET buffer, pH 7.4 (ET buffer: 0.001 M ethylenediaminetetraacetate-0.01 M Tris-HCl) was incubated with ten volumes of DMSO at 37°C for 30 min. After the incubation mixture was dialyzed against 0.1 M NaCl-ET buffer (pH 6.85), ethanol was added to the dialysate to give the final concentration of 35%. Then, the solution containing 35% ethanol was applied onto a Whatman CF-11 cellulose column (1.0 × 10 cm) equilibrated with 0.1 M NaCl-ET buffer (pH 6.85) containing 35% ethanol. The single stranded RDV-RNA fraction was eluted with 0.1 M NaCl-ET buffer (pH 6.85) containing 15% ethanol (Robertson et al. 1968).

The quantitative C'-fixation measurements were made by a modification of Kolmer's micromethod (cf. Ref. 18). Double diffusion tests were carried out at room

temperature in Petri dishes as previously described (Kitagawa and Okuhara 1975 b). RNase A (EC 2.7.7.17) was purchased from Worthington Biochem. Corp. (USA); Pronase E from E. Merck, Darmstadt (Germany). Sephadex G-200 was obtained from Pharmacia (Uppsala, Sweden); Whatman cellulose powder, CF-11, from W. and R. Balston, Ltd. (England). DNA from salmon sperm, Yoshida sarcoma and rat kidney were prepared by the SDS-phenol treatment (Okuhara 1970). Baker's yeast tRNA and rRNA, *E. coli* rRNA and homopolymers of riboadenylate, ribouridylylate, riboinosinate and ribocytidylate (poly (A), poly (U), poly (I) and poly (C)) were obtained from Miles Laboratories, Inc. (Elkhart, Indiana, USA). *Candida utilis* tRNA was kindly supplied by Jujo Paper Manufacturing Co. Ltd. (Akita, Japan) and was further purified by Sephadex G-200 column chromatography. 5 S RNA was isolated from *Candida utilis* tRNA preparation according to the method of Morell et al. (1967).

RESULTS

Serological properties of anti-RDV-RNA sera

Two rabbits were immunized. When 100 ng of RDV-RNA were used as antigen, the titers obtained for CH_{50} with sera of rabbits Nos. 39 and 40 were 340 and 100, respectively. Fig. 1 illustrates the reactivity of various nucleic acids with the diluted (1:50) antiserum No. 39. RDV-RNA, poly (A)·poly (U) and poly (I)·poly (C) reacted similarly with the antiserum, but in the region of antigen excess, the reactivities of poly (A)·poly (U) and poly (I)·poly (C) decreased. Poly (A), poly (U), poly (I), poly (C), native and denatured DNA (salmon sperm, Yoshida sarcoma, rat kidney), tRNA (*E. coli*, *Candida utilis*), rRNA (*E. coli*, baker's yeast), 5 S RNA (*Candida utilis*) and rice plant DNA, rRNA and tRNA did not react at all.

Fig. 1. C'-fixation reactions of the anti-RDV-RNA serum with RDV-RNA and various nucleic acids. (●—●) RDV-RNA; (○---○) poly(A)·poly(U); (Δ---Δ) poly(I)·poly(C); (▲---▲) poly(A), poly(U), poly(I), poly(C), native and denatured DNA (salmon sperm, Yoshida sarcoma, rat kidney, rice plant), rRNA (*E. coli*, baker's yeast, rice plant), tRNA (*E. coli*, *Candida utilis*, rice plant), 5 S RNA (*Candida utilis*)

RDV-RNA reactive antibodies

Fig. 2 shows the precipitating patterns of anti-RDV-RNA antibodies with a variety of antigens. As shown in Fig. 2, two precipitating lines were formed between anti-RDV-RNA antibodies and the corresponding antigen, but one of the

Fig. 2. Double diffusion tests of anti-RDV-RNA serum (Ab) with RDV-RNA (RR), poly(A)·poly(U) (AU) and poly(I)·poly(C) (IC). "Ab"-well contained 30 μ l of the undiluted serum. The peripheral wells contained 40 μ g of each antigen, respectively.

two was connected with a continuous single line formed between anti-RDV-RNA antibodies, poly(A)·poly(U) and poly(I)·poly(C). When RDV-RNA after digestion with RNase A was put in "RR"-well, two precipitating lines were not formed, meaning that two precipitating lines depended on RDV-RNA.

Effect of RNase on RDV-RNA and its antigenicity

As seen in Fig. 3, the antigenic activity of purified RDV-RNA preparation was completely lost after the RNase A treatment in the diluted (1:100) SSC, but not in the undiluted SSC (SSC: 0.15 M NaCl plus 0.015 M sodium citrate). These results obviously indicate that double-stranded RDV-RNA is a true antigen.

C'-fixation reactivities of DMSO-treated RDV-RNA to the antiserum are shown in Fig. 4. First, RDV-RNA was denatured with DMSO and then fractionated with 0.1 M NaCl-ET buffer (pH 6.85) containing 15% ethanol on a cellulose column. As

Fig. 3. Effect of RNase digestion on the C'-fixation of purified RDV-RNA with anti-RDV-RNA serum. RDV-RNA (20 μ g/ml) was digested with RNase A (2 μ g/ml) in the undiluted SSC (○---○) and the diluted (1:100) SSC (▲---▲) for 30 min at 37°C. (●—●) untreated.

Fig. 4. C'-fixation of DMSO-treated RDV-RNA with the antiserum. DMSO-treated RDV-RNA was fractionated on a cellulose column (○---○). DMSO-treated RDV-RNA (20 μ g/ml) was digested with RNase A (2 μ g/ml) in the undiluted SSC (Δ --- Δ) and the diluted (1:100) SSC ($\blacktriangle$ --- $\blacktriangle$). (●—●) untreated.

seen in Fig. 4, its reactivity decreased remarkably but it was not lost completely. Therefore, RNase A digestion of the DMSO-treated antigen was attempted at both high and low salt concentrations in order to examine the double-strandedness of the antigen. As shown in the same figure, the RNA which was digested with RNase A after DMSO treatment had C'-fixation reactivity at high ionic strength, but at low ionic strength the reactivity was completely lost. It indicates that the antigen which reacted with the antiserum was double-stranded RDV-RNA. Therefore, it may at least be said that antibodies elicited in rabbits were not directed toward single-stranded RDV-RNA.

Fig. 5. Antibody activities of IgM and IgG fractionated by Sephadex G-200 column chromatography of anti-RDV-RNA antisera of rabbit Nos. 39 and 40. A column (2.5 $\times$ 45 cm) applied with 2 ml of the antiserum was eluted with 0.05 M phosphate buffer saline (pH 7.3) at 4°C. 3 ml fractions were collected at a flow rate of 10 ml/hr.

Immunoglobulin classes of anti-RDV-RNA antibodies

The immunoglobulin classes of anti-RDV-RNA antibodies were determined using the standard gel filtration technique (Matsumoto and Okuhara 1974). As shown in Fig. 5, the antibodies to RDV-RNA in sera from rabbits No. 39 and No. 40 were IgG and IgM, respectively.

DISCUSSION

Naturally occurring double-stranded RNA of plant virus, RDV-RNA, was demonstrated to have immunogenicity in rabbits. Anti-RDV-RNA antibodies cross-reacted strongly with poly (A)·poly (U) or poly (I)·poly (C). The effect of RNase digestion of RDV-RNA on the C'-fixation (Fig. 3) provided a strong evidence that the antibodies produced are directed to double-stranded RDV-RNA.

In double diffusion tests we observed two different precipitation lines formed between RDV-RNA and anti-RDV-RNA antibodies (Fig. 2). One of the two precipitation lines was connected with that formed between poly (A)·poly (U), poly (I)·poly (C) and anti-RDV-RNA antibodies. This represents the presence of the cross-reacting antibodies probably common to the double helical structure of poly (A)·poly (U), poly (I)·poly (C) and RDV-RNA. As far as an additional precipitation line which appeared between the antiserum and RDV-RNA is concerned, it was thought to stand for specific antibodies to the unique structure of RDV-RNA. We have attempted to demonstrate by means of absorption test whether the additional line represents the presence of specific antibodies or not. "Cross-reacting antibodies" present in anti-RDV-RNA sera were absorbed with poly (I)·poly (C) and/or poly (A)·poly (U), and double diffusion tests among poly (A)·poly (U), poly (I)·poly (C), RDV-RNA and the absorbed serum were performed, but the "specific" precipitation line was hardly detected in agar gel. However, considering the data obtained in the experiments, it seems most likely that anti-RDV-RNA sera might contain the antibodies to the unique structure of RDV-RNA.

In our previous paper we stated that the antibodies formed in rabbits hyperimmunized with poly (A)·poly (U) or poly (I)·poly (C)-MBSA complexes showed potent activities in the IgG fraction of immunoglobulins (Matsumoto and Okuhara 1964), whereas, in the case of the immunization with RDV-RNA-MBSA complexes, the antibody activities were demonstrable in both IgM and IgG.

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