Aggregation Ability of Virus-Specific Antibodies is Correlated with their Capacity to Neutralize *Rice dwarf virus*

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Abstract

Antibodies (immunoglobulin G (IgGs)) from antisera raised against viral particles that had been dissociated by treatment with SDS and intact particles of *Rice dwarf virus* (RDV) were studied for their ability to prevent viral infection of vector cells in monolayers *in vitro*. Even though IgGs raised against dissociated virus had a higher titer than those raised against intact viruses in an analysis of viral proteins on Western blots, they did not neutralize RDV. Conversely, IgGs raised against intact RDV effectively neutralized viral infectivity. Electron microscopic observation of the aggregation of RDV particles after incubation with IgGs raised against intact RDV, but no aggregation of RDV particles after incubation with IgGs raised against dissociated RDV suggested that IgGs raised against intact viruses might prevent viral invasion by causing clumping of viruses, thereby reducing the number of infectious units. Our results reveal, for the first time, a possible mechanism for the neutralization, mediated by antibodies, of plant viruses that propagate in insect vector cells.

Introduction

Neutralizing antibodies act, in part, by blocking both the binding of viruses to permissive cells and their entry into the same cells. In general, such antibodies bind to accessible surface determinants on virions to prevent infection[3,5,11,19]. The neutralization of the infectivity of animal viruses has been studied extensively by the plaque assay technique. Conversely, few such neutralization studies have been performed with plant viruses[1,4,6,7,8,12,17,19]. The difference is due mainly to the lack of sensitivity and precision of assays used to measure the infectivity of plant viruses[20].

The development of techniques for the culture of vector cells in monolayers (VCMs) and the use of immunofluorescence techniques for counting infected cells has provided a high degree of experimental control and accuracy for neutralization experiments with plant viruses that propagate in their respective vectors[9,10]. *Rice dwarf virus* (RDV), which belongs to the genus *Phytorerovirus* in the family *Reoviridae*[2], is unique insofar as it is able to multiply both in plants and insect vectors[2], and leafhopper VCMs have proved an excellent system for RDV[9] studies. Moreover, complete infection of cells can be achieved with a highly diluted inoculum, allowing the synchronous multiplication of the virus to be monitored in detail[9,22]. Thus, neutralization studies using such VCMs are quantitative and reproducible.

In the present study, we analyzed the effects on the

RDV infectivity of antibodies (immunoglobulin G (IgGs)) from antisera raised against viral particles that had been dissociated by SDS and intact particles of RDV. Subsequently, we examined whether the qualitative properties of the binding of antibodies to virus particles could account for the observed difference in the ability to neutralize viruses *in vitro*. Our evaluation of the ability of the aggregation of antibodies of RDV particles is relevant to the neutralization potential of the antibodies and demonstrates that differences in the aggregation kinetics of antibodies of RDV particles correlate to neutralization capacity.

Materials and methods

1. Cells, virus and antibodies

NC-24 cells, originally established from embryonic fragments that had been dissected from eggs of *Nephotettix cincticeps*, were maintained in a monolayer culture at 25°C in a growth medium that had been prepared as described earlier[10].

IgGs raised in rabbits against intact RDV, and RDV particles, from the same batch, that had been dissociated by SDS[13] were used in this study. The RDV had been purified from infected rice plants, basically as described earlier[15]. Each particle was composed of six structural proteins, namely, the P1, P5 and P7 core proteins enclosed by P3, the major core capsid protein plus the P8 major and P9 minor outer-capsid proteins[14,16,22,23]. The total amount of viral antigen used was almost identical for both types of immunization, and IgGs were isolated from each preparation of antiserum by affinity chromatography on a column of protein A-Sepharose (Pierce, Rockford, USA). Eluted IgGs were dialyzed exhaustively against phosphate-buffered saline (PBS).

2. SDS-PAGE and Western blotting analyses

Twofold diluted solutions of purified viral particles (167 mg/ml) were subjected to SDS-PAGE (12% polyacrylamide). Subsequently, viral proteins were transferred from the gel to a polyvinylidene difluoride membrane (Millipore). The membrane was blocked with 3% BSA in PBS that contained 0.05% Tween 20 at 4°C overnight. The membrane was then incubated with a 5,000-fold-diluted solution of IgGs raised against intact RDV or IgGs raised against dissociated RDV (1 mg/ml) and processed as described earlier[23].

3. Neutralization of RDV

Virus-neutralization assays were performed with VCMs by determining the concentration of antibodies capable of reducing the infectivity of viruses at a fixed concentration (3.35 μg/ml) by 50%. Serial five-fold dilutions of each preparation of antibodies were incubated with a solution of viruses at 3.35 μg/ml in His-Mg for 1 h at 37°C. Subsequently, the incubated mixtures were added to VCMs and incubation was continued for 2 h at 25°C, whereupon the mixtures were removed from VCMs and the cells were gently washed several times with His-Mg at room temperature. Each monolayer was then covered with 0.1 ml of medium and incubated at 25°C for 48 h. VCMs were washed with PBS and fixed for 30 min at room temperature in 2% paraformaldehyde. Fixed cells were washed with PBS and permeabilized in PBS that contained 1% BSA and 0.1% Triton X-100, whereupon cells were washed with PBS and incubated with a 100 fold-diluted solution of viral particle-specific IgGs that had been conjugated directly to fluorescein isothiocyanate (FITC) for 1.0 h at 37°C. Finally, VCMs on coverslips were washed with deionized water and mounted on glass slides with ProLong Antifade (Invitrogen). Cells were visualized under a Zeiss 510 confocal laser-scanning microscope (LSM). Neutralizing titers were recorded as the concentration (μg/ml) of antibodies that reduced viral infection by 50%. The values reported represent averaged results of at least three independent experiments.

4. ELISAs

We determined the binding of antibodies to intact viral particles by an enzyme-linked immunosorbent assay (ELISA). In brief, microtiter wells were coated with viral particles by incubating 50 μl of a solution of RDV at a concentration of 2.5 μg/ml diluted by 0.02 mol/L PBS (pH 7.3) in each well overnight at 4°C. After washing with 0.02% Tween 20 in PBS, all wells were blocked with 3% BSA in PBS before incubation with 10-fold serial dilutions of antibodies for 1 h at 37°C. The wells were washed three times, then goat antibodies against rabbit IgGs conjugated with alkaline phosphatase (Sigma-Aldrich) diluted 1:500 in PBS that contained 1% BSA, were added and the plate was incubated for 1 h at 37°C. The wells were washed three times with PBS and the reaction was visualized by the addition of 50 μl of an alkaline phosphatase substrate solution. After 30 min at 37°C, the optical density at 405 nm was determined with a microplate reader (Bio-Rad).

5. Surface plasmon resonance

Surface Plasmon resonance (SPR) is a label-free technology for monitoring biomolecular interactions in real-time. It measures mass changes induced by the association or dissociation of an immobilized ligand and a binding analyte. Interactions between antibodies and viral particles can be identified and quantitated by SPR us-

ing the BIAcore 2000 and BIAcore 3000 systems (GE Healthcare, Buckinghamshire, UK)[18]. Eight thousand response units (RU) of IgGs raised against either intact RDV or dissociated RDV, diluted in 10 mM sodium acetate (pH 4.5), were immobilized on the dextran matrix of a CM5 sensor chip using an amine coupling kit in accordance with the manufacturer's instructions. The resultant surface densities ranged from approximately 5,000 to 6,000 RU. Real-time interactions were analyzed by injecting viral particles at 10 μg/ml. Binding experiments were performed in HEPES-buffered saline [10 mM HEPES (pH 7.4), 150 mM NaCl] at 25°C and at a flow rate of 30 μl/min. Pulses of 5 mM NaOH were used to regenerate the surface of the chip. A blank flow cell served as a negative control. Binding curves were processed with BIAevaluation 3.1 software (GE Healthcare).

6. Electron microscopy

Purified RDV were diluted in His-Mg to a concentration of 3.35 μg/ml. A range of concentrations of antibodies from 200 to 0.02 μg/ml was prepared by making basically 5-fold dilutions of antibodies in His-Mg. Equal volumes (100 μl) of viral suspension and diluted antibodies were combined and incubated for 1 h at 37°C. Aliquots (2 μl) of each mixture were applied to carbon-coated grids, negatively stained with 2% uranyl acetate, and examined with an electron microscope (H-7000; Hitachi, Naka, Japan).

Results

1. Reactions of IgGs raised against intact RDV and IgGs raised against dissociated RDV with viral structural proteins, as determined by Western blotting

Two-fold-diluted solutions of purified RDV were subjected to SDS-PAGE and the reactivity of the two preparations of IgGs was analyzed by Western blotting. Antibodies raised against intact viruses detected the major outer capsid protein P8, the minor outer capsid protein P9 and the core capsid protein P7; antibodies raised against dissociated RDV detected the core proteins P1, P3 and P5 in addition to those detected by the antibodies raised against intact RDV (data not shown). As shown in Fig. 1, IgGs raised against intact RDV clearly reacted with P8 up to 8,192-fold dilution while IgG raised against dissociated RDV reacted up to 16,384-fold dilution. A similar difference in reactivity was observed in the detection of P7 and P9 with the same two preparations of antibodies (data not shown). The dilution end-point for a positive reaction with IgGs raised against intact RDV with
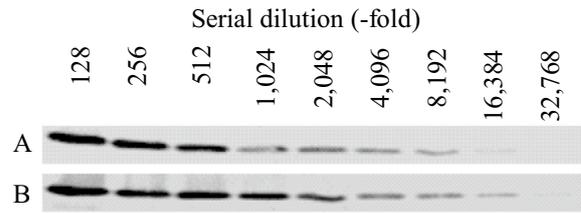

Fig. 1. Western blotting analysis of the reactions of IgGs raised against intact RDV and IgGs raised against dissociated RDV with the outer-capsid protein P8 of RDV

Two-fold serially diluted solutions of purified virus (167 mg/ml), as indicated, were fractioned by SDS-PAGE. Proteins were transferred to membranes and probed with IgGs raised against intact RDV (A) and IgGs raised against dissociated RDV (B), as indicated.

P8 was half that for the IgGs prepared against dissociated RDV. Similar results were obtained using different lots of purified RDV.

2. IgGs raised against intact RDV, but not IgGs raised against dissociated RDV, effectively neutralized RDV

To determine the effects on RDV infectivity of IgGs raised against intact RDV and IgGs raised against dissociated RDV, we examined the neutralizing effects of the RDV-antibodies using a standard neutralization assay. As shown in Fig. 2, IgGs raised against intact RDV had significant neutralizing activity against RDV. The neutralizing titer of IgGs raised against intact RDV, measured in terms of the 50% inhibition of RDV infection, was 0.05 μg/ml. Conversely, IgGs raised against dissociated RDV had no neutralizing activity, even at 100 μg/ml.

3. Both IgGs raised against intact RDV and IgGs raised against dissociated RDV bound viral particles in an ELISA

The ELISA is a well-characterized assay for monitoring the binding of antibodies to a given antigen. In the current study, we measured the relative affinity of IgGs raised against intact RDV and IgGs raised against dissociated RDV for viral particles by ELISA. As shown in Fig. 3, both IgGs raised against intact RDV and IgGs raised against dissociated RDV bound to viral particles. However, the relative affinity of IgGs raised against intact RDV for viral particles was as much as 10-fold higher than that of IgGs raised against dissociated RDV. Similar results were obtained to detect viruses in infected plants (data not shown). Therefore, the relative affinity, as measured by ELISA, failed to account for the differ-
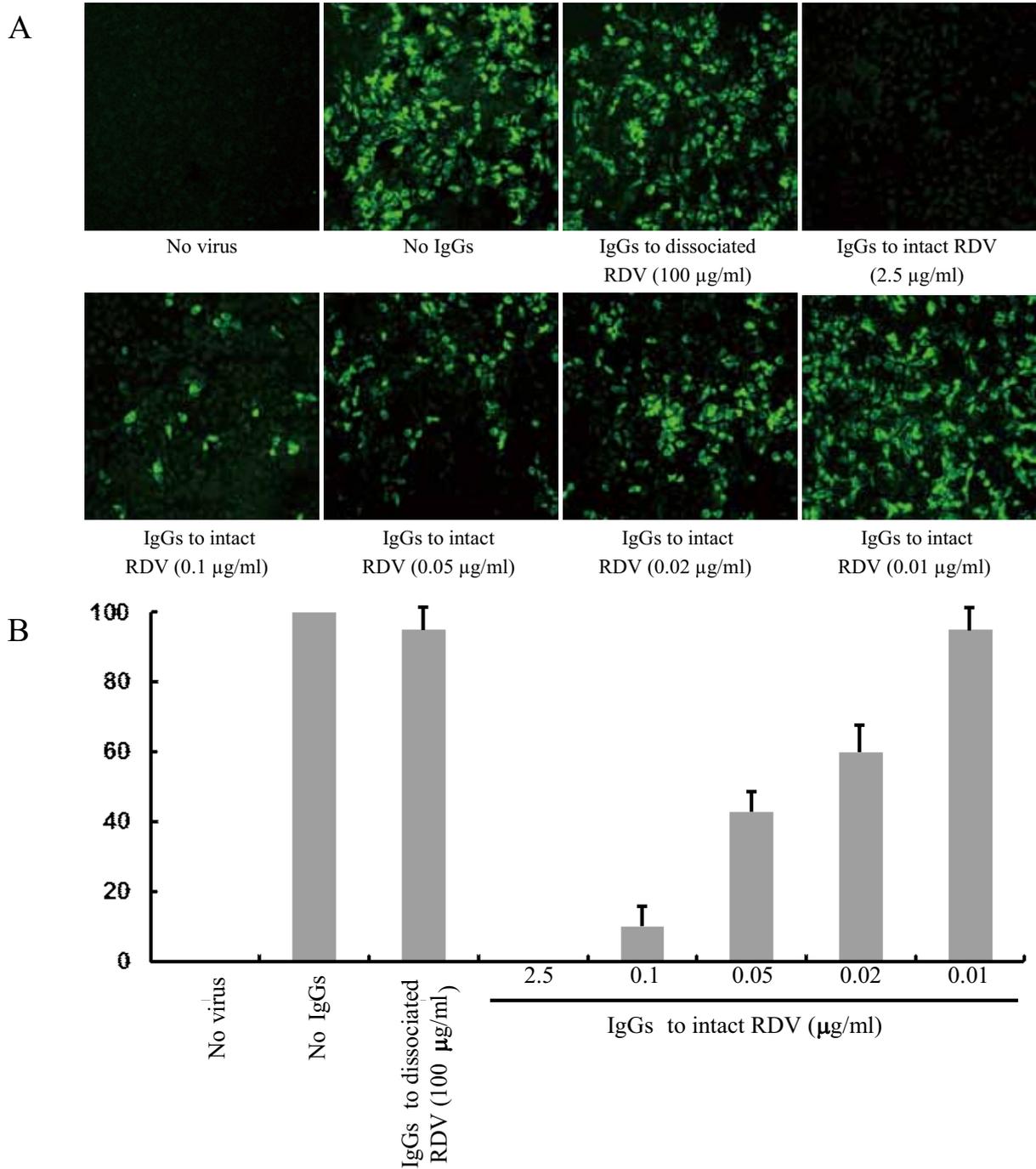
Fig. 2. **The neutralizing effects of IgGs raised against intact RDV and IgGs raised against dissociated RDV on infection of VCMs by RDV**

(A) Results from one neutralization experiment are shown. (B) Relative virus infection of cells inoculated with RDV mixed with IgGs. Each column and bar in the histogram represent the percentage of infected cells in each field of view, calculated as the average of values from at least four non-overlapping random fields of cells from each of three independent experiments. The error bars indicate the standard deviation.

ence that we observed in the abilities of IgGs raised against intact RDV and IgGs raised against dissociated RDV to reduce viral infectivity.

4. Comparison of the binding to RDV of IgGs raised against intact and dissociated viral particles by surface plasmon resonance (SPR)

We characterized the ability of the two preparations of IgGs to bind RDV in "real-time" using SPR. We immobilized IgGs on the CM5 sensor chip then flooded the prepared surface with intact RDV. Values from the nega-
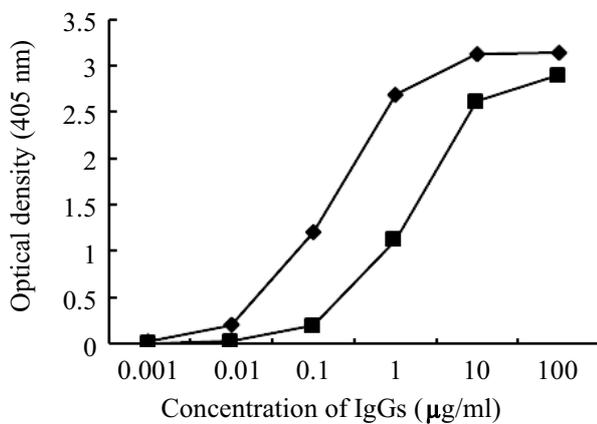

Fig. 3. Binding of intact viral particles to IgGs raised against intact RDV (◆) and IgGs raised against dissociated RDV (■), as determined by ELISA
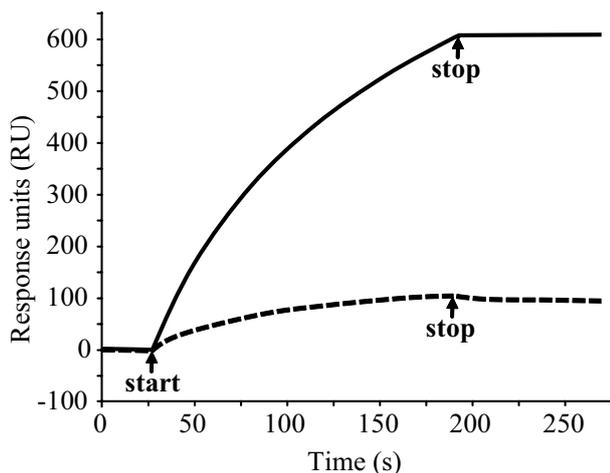

Fig. 4. Biacore SPR analysis of the interactions of viral particles with antibodies
Sensorgrams show real-time interactions between immobilized IgGs (raised against dissociated RDV, broken line; and raised against intact RDV, solid line) and purified RDV particles (10 μg/ml). Arrows indicate the end of the sample injections and the beginning of the dissociation phase initiated with the running buffer.
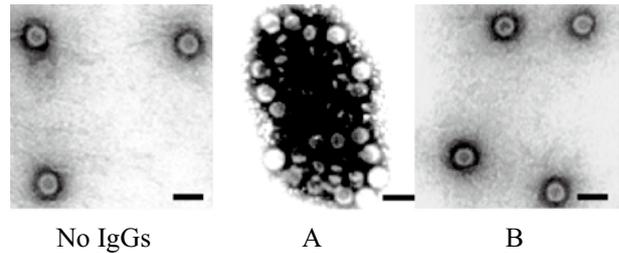

No IgGs   A   B

Fig. 5. Electron microscopic analysis of RDV-antibody complexes
Purified RDV (3.35 μg/ml) was mixed with IgGs raised against intact RDV (0.05 mg/ml) (A) or IgGs raised against dissociated RDV (100 mg/ml) (B). Bars, 100 nm.

tive control were subtracted from the resultant sensorgrams. As shown in Fig. 4, RDV associated with both types of IgG, with little dissociation. The binding value to RDV of IgGs raised against intact RDV was approximately six times higher than that of IgGs raised against dissociated RDV. Relative affinity, as measured by this method, also failed to account for the difference that we observed in the abilities of IgGs raised against intact RDV and IgGs raised against dissociated RDV to reduce viral infectivity.

5. Electron microscopic analysis of RDV-antibody complexes

Since IgGs raised against intact RDV and IgGs raised against dissociated RDV differed significantly in terms of abilities to neutralize RDV, and the difference was not explained by ELISA and SPR, we then examined virus-antibody interactions by electron microscopy. As shown in Fig. 5, no clumping or aggregation of viral particles was observed after incubation of RDV particles with IgGs raised against dissociated RDV, even at a concentration of IgGs of 100 μg/ml. Conversely, conspicuous clumping or aggregation of viral particles was observed over a range of dilutions of IgGs raised against intact RDV. Aggregates of small numbers of viral particles were observed even at antibody concentrations as low as 0.02 μg/ml.

Discussion

IgGs raised against dissociated RDV had a higher titer than those raised against intact RDV in reactions with viral proteins on Western blots. This result was reasonable because the antigen used for detection on Western blots had been dissociated from viral particles by SDS during PAGE, and the IgGs raised against dissociated

RDV used to detect antigens had been raised against SDS-treated viral particles. This scenario implies that IgGs raised against dissociated RDV includes those against the cryptotopes of viral particles[13,20,21] in addition to those against polypeptides that are located on the outer face of P8 in particles of RDV. Thus, antibodies prepared against SDS-dissociated virions proved sensitive tools for detecting viral antigens on Western blots.

Conversely, IgGs raised against dissociated RDV did not neutralize RDV, even at 100 μg/ml, while IgGs raised against intact RDV at 0.05 μg/ml caused a 50% inhibition of RDV infection. This difference in neutralizing effects suggested that binding of IgGs raised against intact RDV to intact viral particles differed from that of IgGs raised against dissociated RDV. Therefore, we examined the mechanisms responsible for this difference by ELISA, an examination of viral reaction, and SPR. ELISAs showed that IgGs raised against intact RDV had a higher affinity for intact virus particles than those raised against dissociated RDV. However, this observation did not explain the difference between the results of the neutralization study. Furthermore, relative affinity, six times higher in IgGs raised against intact RDV than that of IgG raised against dissociated RDV, as measured by SPR, also failed to account for the difference that we observed in the abilities of IgGs raised against intact RDV and IgGs raised against dissociated RDV to reduce viral infectivity.

Electron microscopic analysis of viral aggregation in the presence of IgGs clearly revealed the difference between the antibodies in terms of the ability to aggregate RDV. IgGs raised against dissociated RDV did not cause clumping of intact viral particles, even at a concentration of IgG of 100 μg/ml, but IgGs raised against intact RDV retained the ability to cause aggregation, even at 0.02 μg/ml. The coincidence of this ability to generate aggregates and the ability to neutralize the virus suggested that the aggregation ability, rather than reactivity, defines the neutralization ability of the antibodies. Thus, IgGs raised against intact RDV appeared to prevent cell invasion by blocking the adsorption of virus particles to its target cells via clumping of viruses that reduced the number of infectious units available for cell invasion.

Acknowledgments

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