Anti-Idiotype Antibodies
Against an Anti-Barley Yellow Dwarf Virus Monoclonal Antibody

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ABSTRACT


Antisera were produced in rabbits against purified anti-RPV monoclonal antibody (mAB-RPV1), which neutralizes RPV transmission by Rhopalosiphum padi. Active anti-idiotype antibodies were purified from the rabbit antiserum with a protein A-Sepharose column. After immunoprecipitation with both mouse serum and mouse IgG, the antibodies reacted with mAB-RPV1 but not with several other anti-RPV monoclonal antibodies of the same subclass. The antibodies inhibited reactions between RPV and mAB-RPV1; the reactions between mAB-RPV1 and the antibodies also were inhibited by RPV in competitive enzyme immunosorbent assays. When the antibodies were injected into a rabbit, active anti-RPV (=anti-anti-idiotype) antiserum was produced. We conclude that true anti-idiotype antibodies against mAB-RPV1 were produced.

Additional keywords: aphid vector, virus-vector specificity.

Barley yellow dwarf virus (BYDV), type member of the plant luteovirus group, is an isometric, single-stranded RNA virus (26). This virus, which is phloem limited, is transmitted in a persistent-circulative manner by aphids (26). Five distinct isolates of BYDV, transmitted in a vector-specific manner by four aphid species, have been characterized at Cornell University, Ithaca, NY (26). One goal in our laboratory was to understand the mechanism of this virus-vector specificity. Our current working hypothesis for the specificity is that interaction of one or more epitopes on the virus protein capsid with receptors on membranes of aphid accessory salivary glands determines which virus isolate an aphid can transmit (10). Previous studies (8,9) have provided evidence that the plasma membrane of aphid accessory salivary glands is the selective barrier that controls the specificity of virus transmission by aphids. If the epitope-receptor hypothesis is correct, the putative receptors, which regulate virus uptake, should be on the plasma membrane. However, we do not have any direct evidence for the receptors. It is very difficult to study the putative receptors directly because aphids are such small insects. Each aphid has only two accessory salivary glands and each gland is composed of only four cells (20). Therefore, we tried a novel technique from immunology: use of anti-idiotype antibody to study cell surface receptors (5,28).

Epitopes associated with antigen-binding sites on antibodies are called idiotypes. Antibodies against the idiotypes are anti-idiotype antibodies (6). The idea of using anti-idiotype antibody to study receptors on membranes (Fig. 1) (14) is to make primary antibody against the virus epitope, which specifically reacts with the receptor on the membrane. Then the primary antibody is used as immunogen to make a second antibody. If the antibody to the virus and the receptor are equivalent, the anti-idiotype antibody will be equivalent to the virus epitope and should react with the receptor. The most important advantage of using this technique is that we can make an antibody that reacts with a receptor without having to isolate the receptor. Others have used this technique successfully to characterize and isolate various receptors (6), such as mammalian receptor (3), reovirus receptors (3).

The objectives of this study were to produce anti-idiotype antibody to an anti-BYDV monoclonal antibody (mAB), try the anti-idiotype antibody as a probe to identify the putative receptors on aphid salivary glands, and prime and elicit anti-anti-idiotype antibody (= anti-virus antibody). Here we report work on production and characterization of the anti-idiotype antibody and anti-anti-idiotype antibody. Potential application of anti-idiotype antibody technique in plant virology is discussed. A preliminary report has been published (13).

MATERIALS AND METHODS

The RPV isolate of BYDV, transmitted by Rhopalosiphum padi (L.), was used (22,25). Stock colonies of the same clone of the aphid species used previously were maintained on barley as described (22). Coast Black oats (Avena byzantina K.) were test plants in all bioassays. Clarified virus preparations were made by grinding tissue with a Brinkmann polytron homogenizer (Brinkmann Instruments Co., Westbury, NY) and mixing it with chloroform (23). Purified virus was made as previously described by chloroform clarification, differential centrifugation, and sucrose gradient centrifugation (24). Enzyme immunosorbent assay (EIA) was carried out primarily with the two-day procedure (23), unless described otherwise. Anti-BYDV polyonal antibodies were produced in rabbits (25).

Comparison of epitope specificity of monoclonal antibodies.
Three anti-RPV monoclonal antibodies, mAB-RPV1 (IgG2a), mAB-RPV2 (IgM), mAB-RPV3 (IgG2a), had been produced (12). They all react with RPV but not with other BYDV isolates. To determine their epitope specificity, the mABs, purified from ascitic fluid by affinity chromatography on a protein A-Sepharose column (Pharmacia Fine Chemicals, Piscataway, NJ), were labeled with biotin and used in competitive EIA. For labeling, 2 mg
of the mAB in 1 ml of 0.1 M NaHCO₃ and 0.15 M NaCl was mixed with one-tenth volume of a fresh solution of N-hydroxysuccinimido-biotin (obtained as a powder from Sigma, St. Louis, MO), which was 2.5 mg/ml of dimethyl sulfoxide. The mixture was allowed to react for 2 hr at room temperature and dialyzed against several changes of 0.05 M phosphate buffered saline (PBS), pH 7.2, overnight (30).

In competitive EIA (30), polyclonal microtiter plates (Dynatech Laboratories, Inc., Alexandria, VA) were coated with anti-RPV polyclonal antibodies at 37 C for 4 hr; then partially purified RPV was added and incubated at 4 C overnight, using a predetermined dilution of virus, which ensured that the amount of unlabeled antibody was limiting when added later. After unlabeled mABs had been added to wells at different dilutions and incubated for 3 hr at 37 C, the biotin-labeled mABs were added to the wells and incubated for 3 hr at 37 C. Avidin-phosphatase conjugate (Sigma) was used at 1:2,000 dilution to react with biotin for 3 hr at 37 C. Finally, the wells were incubated with p-nitrophenyl phosphate substrate at room temperature. In each test, a homologous competitor was included as a positive control, and bovine serum albumin was used as a negative control. Percentage of inhibition by competitor mABs in the reaction of RPV with labeled mABs was calculated with the following equation:

\[
\text{% of inhibition} = \left(\frac{\text{O.D. of positive control} - \text{O.D. of sample}}{\text{O.D. of positive control}}\right) \times 100
\]

Neutralization studies. *R. padi* aphids were injected with mAB-RPV1, mAB-RPV2, and mAB-RPV3 to assess the neutralization ability of these antibodies as described previously (16). The monoclonal antibodies (ascitic fluid) were diluted 1:25 in 0.01 M PBS, pH 7.0. About 0.02 μl of the diluted ascitic fluid was injected into each aphid. Injected aphids were allowed to recover at room temperature from CO₂ anesthesia and then placed on RPV-infected oat leaves for a one-day acquisition period at 15 C. Single aphids then were transferred to oat seedlings for a 4-day inoculation test feeding at 21 C. Plants were fumigated and kept in a greenhouse (22). Infection of plants during the following 4–5 wk was determined by observation of symptoms.

Production of anti-idiotypic antibodies. Protein A-Sepharose column-purified mAB-RPV1 (100 μg in 0.5 ml of PBS emulsified in 0.5 ml of incomplete Freund's adjuvant) was injected intradermally at multiple sites on the backs of each of three Flemish Giant rabbits every 2 wk (29). The rabbits were bled 1 wk after the third injection. Double sandwich EIA was used to monitor the rabbit polyclonal antibody titers. Microtiter EIA plates were coated with mAB-RPV1 (1:1,000 of ascitic fluid) for 4 hr at 37 C; different dilutions of the rabbit antisera were incubated for 4 hr at 37 C; and then goat-anti-rabbit conjugate (Sigma) was placed in wells for 4 hr at 37 C. Antibodies against nonidiotype epitopes were removed by immunoprecipitation (500 μl of anti-idiotypic rabbit serum + 100 μl of mouse serum + 1 mg of mouse IgG in 100 μl PBS, Sigma) at 37 C for 1 hr and then at 4 C overnight. The IgG was isolated from the absorbed antisera by protein A-Sepharose chromatography (11).

Production of anti-anti-idiotypic antibodies. A Flemish Giant rabbit was immunized intramuscularly with 600 μg of protein A-Sepharose column-purified anti-idiotypic IgG, which had been emulsified in an equal volume of Freund's incomplete adjuvant. Two more injections of the purified IgG in the same adjuvant were given on day 7 and day 14, using 400 μg and 160 μg, respectively. One month after the last injection, a booster was given by injecting 500 μg of immunogen in incomplete adjuvant. Blood samples were collected and checked for anti-RPV activity. Aliquots (0.4 ml) of clarified RPV preparations were mixed with 0.1 ml of the antisera taken at different dilutions at 37 C for 1 hr and then at 4 C for 3 hr. Preimmunization serum from the same rabbit was used as a control. The virus-antibody mixtures were assayed by the RPV homologous direct EIA for unreacted virus. The antisera also were tested for neutralization activity on virus transmission in injection bioassay (as described in the section on neutralization studies) and in membrane feeding bioassay. The membrane feeding assay, clarified RPV preparations were mixed in test tubes with the antisera (anti-anti-idiotypic antibodies), with preimmunization serum, anti-RPV antibodies, or 0.01 M potassium phosphate buffer as the control. Each mixture was kept at 37 C for 1 hr and then diluted with an equal volume of 40% sucrose in 0.01 M phosphate buffer. After aphids had fed through stretched Parafilm on the treated virus preparations for 24 hr at 15 C, they were moved to oat seedlings (10 aphids per seedling) for 5 days at 21 C. Plants then were treated in the same manner as the plants in the injection assays.

RESULTS

Epitope specificity. Results from the competition EIA, in which biotin-labeled mAB-RPV1 was used, are shown in Figure 2. Nonlabeled mAB-RPV1 inhibited the reaction between RPV and the labeled mAB-RPV1 completely, even when diluted preparations (1:800) were used. Nonlabeled mAB-RPV3 inhibited the reaction in the range of less than 20%; thus it is different from mAB-RPV1. Nonlabeled mAB-RPV2 inhibited the reaction in the 70–90% range. This high but incomplete inhibition might be due to allosteric hindrance effect. That is, the binding of antibody molecules with virions could cause conformational changes of virus capsid (31), and this change could inhibit binding of other antibody molecules to the virions.

When anti-idiotypic antibody (against mAB-RPV1) was produced, it reacted strongly with mAB-RPV1, not at all with mAB-RPV3, and very weakly with mAB-RPV2 (2). Therefore, these results confirmed that mAB-RPV1, mAB-RPV2, and mAB-RPV3 reacted with different RPV epitopes (12). Two other monoclonal antibodies also were produced previously from the same hybridoma cell lines as mAB-RPV1 and mAB-RPV3. Reactions of these two monoclonal antibodies in the two kinds of experiments were identical with those of mAB-RPV1 and mAB-RPV3, respectively (data not shown).

Neutralization studies. The neutralization action of the anti-RPV monoclonal antibodies on virus transmission was examined by injecting the mAB into aphids. The mAB-RPV1 was found to be
the best neutralizing antibody. It inhibited virus transmission more than 90%; in contrast, mAB-RPV2 and mAB-RPV3 decreased virus transmission about 70 and 25%, respectively. Therefore, mAB-RPV1 was selected for the production of anti-idiotypic antibody.

Production of anti-idiotypic antibodies. Antisera from all three rabbits reacted similarly in characterization tests. Results only for rabbit number 2 are given here. The antisera were precipitated with mouse serum and mouse IgG. Results (Fig. 4) show that, before absorption, the rabbit antisera reacted with both immunogen (mAB-RPV1) and the control, which was anti-RPV mAB-RPV3, the same subclass (IgG2a) as mAB-RPV1 (12). After absorption, the rabbit antisera still reacted with mAB-RPV1, but not with the same subclass control (Fig. 4). Apparently the absorption eliminated most nonspecific antibodies.

If the rabbit antibodies are true anti-idiotypic antibodies, they should carry an image of the virus epitopes and they should inhibit the reaction between RPV and mAB-RPV1. In addition, RPV should inhibit the reaction between mAB-RPV1 (idiotype) and the rabbit antibody (anti-idiotype). Two kinds of tests were done to evaluate these possibilities. First, EIA plates were coated with mAB-RPV1; then the rabbit antibodies were added, then RPV, and finally anti-RPV conjugates. Rabbit antibodies inhibited the reaction between mAB-RPV1 and RPV in a dosage-dependent manner (Fig. 5). In the other type of test, EIA plates were coated with mAB-RPV1, then RPV was added before the rabbit antibodies were used, and finally goat-anti-rabbit-enzyme conjugate was added. Again, RPV inhibited the reaction between mAB-RPV1 and the rabbit antibody in a dosage-dependent manner (Fig. 6).

Production of anti-idiotypic antibodies. Anti-idiotypic antibodies reactive with the antigen-binding sites of anti-RPV monoclonal antibodies may contain subpopulations that mimic the virus epitopes recognized by the monoclonal antibodies (14). Therefore, injection of animals with the anti-idiotypic antibodies

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**Fig. 2.** Epitope specificity study of three anti-RPV monoclonal antibodies (mABs) in a competitive enzyme immunoassay (EIA) that tests the inhibition of reaction between RPV and biotin-labeled mAB-RPV1 by unlabeled mABs. Anti-RPV polyclonal antibodies were coated on EIA plates to trap RPV virions. The immobilized RPV was detected by biotin-labeled anti-RPV monoclonal antibody (mAB-RPV1) after incubation with nonlabeled anti-RPV monoclonal antibodies. Then avidin-enzyme conjugate was added. Finally, substrate was incubated for 45 min at room temperature.

**Fig. 3.** Epitope specificity test of three anti-RPV monoclonal antibodies (mABs) with anti-idiotypic antibodies. The monoclonal antibodies were used to coat enzyme immunoassay plates; then different dilutions of anti-idiotypic antibody were incubated in the wells. The second layer of antibodies was detected with goat-anti-rabbit-phosphatase conjugate. Absorbance values were means of two wells following a 45-min reaction at room temperature.

**Fig. 4.** Specificity test of anti-idiotypic antibody. Monoclonal antibody (mAB) RPV1 and control (mAB-RPV3), which are both IgG2a, were used to coat enzyme immunoassay assay plates. Anti-idiotypic antibody was absorbed with mouse serum and mouse IgG. The absorbed or nonabsorbed anti-idiotypic antibodies were added to the plates. Then goat-anti-rabbit phosphatase conjugate was added. Absorbance values were means of two wells following a 45-min reaction at room temperature.

**Fig. 5.** Inhibition effect of anti-idiotypic antibody on reaction of RPV and anti-RPV monoclonal antibody (mAB-RPV1) tested in a competitive enzyme immunoassay (EIA). The mAB-RPV1 was used to coat EIA plates. The anti-idiotypic antibody or rabbit-anti-mouse IgG control, at a series of dilutions, was added; then an RPV preparation was incubated to react with the coating antibodies. The trapped virions were detected with anti-RPV polyclonal antibody-phosphatase conjugate.

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may induce antibodies that react with native RPV (6). Accordingly, purified anti-idiotypic antibodies were used to immunize a rabbit and the derived sera were tested for anti-RPV activity (Table I). When more concentrated antisera preparations were mixed with the RPV preparations, RPV activity was eliminated, based on evaluation by EIA. When more diluted (1:243) antisera preparations were used, RPV activity was reduced. In contrast, the preimmunization serum preparations did not reduce RPV activity. When such virus-antisera mixtures were used in the membrane feeding assays, virus transmission by aphids was reduced (Fig. 7). Moreover, when the antisera was injected into R. padi aphids, which were then used for virus transmission, the antisera reduced virus transmission (Fig. 7). This effect of the antisera on RPV activity shown in vitro and in vivo indicated that anti-RPV antisera was produced in the rabbit immunized with the anti-idiotypic antibodies. The production of anti-anti-idiotypic antibodies provided additional evidence that the anti-idiotypic antibodies are real.

**DISCUSSION**

Anti-idiotypic antibodies have been shown to be valuable tools for identifying and studying receptors that are difficult to obtain (6). One example is the use of anti-idiotypic antibody to probe cell surface receptors for mammalian reovirus. A monoclonal anti-idiotypic antibody was produced against monoclonal anti-reovirus antibody (18). The anti-idiotypic antibody inhibited binding of purified virus to the anti-viral monoclonal antibody and bound specifically to reovirus receptor-positive cells (4). The anti-idiotypic antibody has been used to isolate receptors from different types of cells; the receptors have been found to be a 67X 10^8 dalton protein (2). The anti-idiotypic antibody also has been used to quantify the number of receptors on target cells; approximately 50,000 to 75,000 receptor sites occur per cell (4).

More than half of the known plant viruses are transmitted by insect vectors. Receptor-mediated transmission mechanisms have been proposed for both persistent plant virus (10) and nonpersistent ones (1). Direct study of the putative insect cell receptor is almost impossible unless some novel techniques are applied. In the present study, we have produced anti-idiotypic antibodies against an anti-RPV mAb. In preliminary studies with the anti-idiotypic antibodies, we have tried to inject the antibody into aphids to inhibit virus transmission and to locate the putative receptors on aphid salivary gland membranes. To date, we have

**Fig. 7.** Test of effect of anti-anti-idiotypic antibodies on RPV transmission in two bioassays. In the membrane feeding assay (I), clarified RPV preparations were mixed with phosphate buffer (A), preimmunization serum (B), anti-anti-idiotypic antisemur (C), or anti-RPV antisemur (D) at 37°C for 1 hr. The mixtures were diluted with 40% sucrose in 0.01 M phosphate buffer. *Rhaphidophora padi* aphids were allowed to feed through stretched Parafilm on the treated virus mixtures for 24 hr and then have a 5-day inoculation test feeding on oat seedlings. In the injection assay (II), third instars of *R. padi* were injected with preimmunization serum (B), anti-anti-idiotypic antisemur (C), or anti-RPV antisemur (D). Noninjected aphids were used as controls (A). The injected aphids were allowed a 1-day acquisition feeding on RPV-infected oat leaves and then a 5-day inoculation test feeding on oat seedlings. Data are means from two tests. Vertical T-bars represent standard errors.

**Reciprocal dilutions of RPV (Log.)**

![Graph showing inhibition effect of RPV on anto-idiotypic antibodies](image)

**Table 1. Test of anti-anti-idiotypic antibodies for anti-RPV activity in immunoassorpt assay**

<table>
<thead>
<tr>
<th>Dilutions of antisera used for absorption</th>
<th>Absorbance at 405 in enzyme immunoassorbent assay, following preabsorption with antibodies shown*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Test I</td>
</tr>
<tr>
<td>Anti-anti-idiotypic</td>
<td>Control antibody</td>
</tr>
<tr>
<td>1:1</td>
<td>0.067</td>
</tr>
<tr>
<td>1:3</td>
<td>0.247</td>
</tr>
<tr>
<td>1:9</td>
<td>0.067</td>
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<tr>
<td>1:27</td>
<td>0.247</td>
</tr>
<tr>
<td>1:81</td>
<td>0.047</td>
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<tr>
<td>1:243</td>
<td>1.129</td>
</tr>
<tr>
<td>No AB CK</td>
<td>1.129</td>
</tr>
</tbody>
</table>

*Anti-anti-idiotypic antisera were produced in a rabbit injected with purified anti-anti-idiotypic IgG. Clarified RPV preparations (0.4 ml) were mixed with 0.1 ml of the antisera preparations at different dilutions at 37°C for 1 hr, 4°C for 3 hr. Preimmunization serum from the same rabbit was used as the control antibody. The virus-antibody mixtures were assayed by RPV homologous enzyme immunoassorbent assay.

*Values are means of two wells following a 45-min reaction at room temperature.
not obtained significant, direct evidence for the reaction of the antibody with the receptors. However, we are still in the very early stage of application of this new technique, and we foresee the anti-idiotypic antibody as a useful tool for plant virologists to understand basic interactions between viruses and their insect vectors. For instance, the technique could be used to study the epitope-receptor model directly. It could be used to determine the location of the receptors: on plasmalemma of accessory salivary glands, on any specific portions of the membrane, on coated vesicles in cytoplasm of the cell, or elsewhere. The technique could be used to study the nature of the receptors. An affinity column could be set up with anti-idiotypic antibodies to specifically concentrate and purify the receptors for biochemical and biophysical studies. The technique also could be used to study developmental biology of the receptors. Perhaps young instars of greenbug aphids Schizaphis graminum have more specific receptors than adults. This could explain why early instars of the greenbugs are more efficient vectors than the adults.

Production of anti-anti-idiotypic antibodies not only provided additional evidence for the nature of the anti-idiotypic antibodies but also suggested a new approach for the production of antibodies against plant viruses that are difficult to obtain. Purification is difficult for many plant viruses, especially for use in production of large amounts of antibody for diagnosis and research. The hybridoma technique is a good approach to get an unlimited supply of the same antibody; however, it involves very tedious work, and monoclonal antibodies may be too specific for routine diagnosis. Therefore, if antibody from a rabbit against a virus is used to make polyclonal anti-idiotype antibody in a rabbit, the anti-idiotype antibodies then could be used as immunogen to make anti-anti-idiotype antibodies, and an unlimited amount of anti-viral antibodies could be produced. This new “vaccine” approach has been used recently in animal virology (21,32). In the present study, the anti-anti-idiotype antiserum titer was low. It might be because the anti-idiotypic and anti-anti-idiotypic antisera were produced in syngeneic (genetically identical) rabbits. Gaulton and Greene (7) observed that significant responses were induced in syngeneic mice only when anti-idiotype antibody was coupled to a carrier protein for immunization.

Production of anti-idiotype antibody is the initial step for all studies with the anti-idiotype antibody. There are many schemes for inducing anti-idiotype antibody. Polyclonal antibodies have been used as immunogens to produce anti-idiotype polyclonal antibodies (27). More recently, monoclonal antibodies have been used to produce anti-idiotype antibody. Phillips (19) and co-workers coupled a purified monoclonal antibody to key-hole limpet hemocyanin by using 0.25% glutaraldehyde and immunized syngeneic mice. Some other investigators used idiotype-producing hybridoma cells as immunogens for the production of monoclonal anti-idiotype antibodies (18). Sluis and co-workers (29) used a monoclonal antibody to prepare anti-idiotype polyclonal antibody and used them successfully in their studies. Each of these schemes has its advantages and disadvantages. Anti-idiotype monoclonal antibodies are very specific to a particular idiotype, but mAB production is very time consuming. On the other hand, anti-idiotype polyclonal antibody is easy to prepare, but the specific anti-idiotype antibodies are only a subset of the whole antibody population. Theoretically, it is best to produce anti-idiotype antibody in syngeneic animals (5). In practice, both xenogeneic and syngeneic anti-idiotype antibodies have been made, even for the same idiotype (17,18).

When monoclonal antibody is used for production of anti-idiotype antibody, selection of immunogen is very important. By and large, virologists select those monoclonal antibodies that can neutralize virus infectivity. Mechanisms of neutralization differ from simple virus aggregation to blocking of specific sites. Some monoclonal antibodies do block the critical epitope that reacts with cellular receptors (15). Therefore, in this study, we selected mAB-RPV1, which inhibits virus transmission by aphids up to 90%, as immunogen for production of anti-idiotype polyclonal antibody. Results from another study demonstrated that mAB-RPV1 does not aggregate RPV (Hu and Rochow, unpublished).

Therefore, it is likely that mAB-RPV1 blocks epitopes on the RPV viroins that are critical for virus transmission. Further studies on the epitopes would provide insightful information on the virus-aphid interaction.

LITERATURE CITED


