Immunological Evidence for a Cell Surface Receptor of Victorin Using Anti-Victorin Anti-Idiotypic Polyclonal Antibodies

Kazuya Akimitsu1,2, L. Patrick Hart1,2, and Jonathan D. Walton1,3

1Department of Botany and Plant Pathology, 2Pesticide Research Center, and 3DOE-Plant Research Laboratory, Michigan State University, East Lansing 48824 U.S.A. Received 9 November 1992. Accepted 9 March 1993.

Anti-victorin anti-idiotypic polyclonal antibodies were raised in rabbits immunized with an anti-victorin polyclonal antibody-ovalbumin conjugate. The presence of anti-victorin anti-idiotypic antibodies in the serum was examined by the inhibition of victorin-horseradish peroxidase (HRP) binding against anti-victorin antibody in a direct enzyme-linked immunosorbent assay (ELISA). Victorin-HRP binding in a direct ELISA was inhibited 84% by addition of a tenfold diluted anti-victorin anti-idiotypic serum. Sera from nonimmunized rabbits did not show significant inhibition. The anti-idiotypic serum diluted 20-fold, induced callose synthesis in protoplasts from victorin-sensitive but not insensitive oat cultivars. When anti-idiotypic antibodies were added simultaneously with 60 pg of victorin per milliliter, they inhibited victorin-induced callose synthesis by 68%. Since anti-idiotypic antibodies are presumed to be too large to enter the cell, these results indicate that victorin acts at the surface of cells.

Cochliobolus victoriae (Meehan & Murphy) Subram & Jain, the fungus that causes victoria blight of oats (Avena sativa L.), produces a host-specific toxin, victorin. Both susceptibility to the pathogen and sensitivity to victorin are determined by the dominant allele of the Vb gene (Pringle and Schaeffer 1964). It has been hypothesized that the Vb gene encodes a receptor for victorin and that lack of a receptor is the cause of resistance (Pringle and Schaeffer 1964).

Several lines of indirect and direct evidence support the existence of a victorin receptor site or sites; pretreatment with inhibitors of RNA or protein synthesis or reagents that block SH groups reduce the sensitivity of oats to victorin (Gardner and Schaeffer 1973; Rancillac et al. 1976; Walton and Earle 1985). Pretreatment with heat also induces the loss of sensitivity to victorin (Briggs et al. 1984; Bronson and Schaeffer 1977). These data indicate that victorin sensitivity requires a constitutive protein, hypothesized as a receptor, with a short turnover time (<12 hr). Wolpert and Macko first reported that an 125I-labeled victorin analog binds covalently to a 100-kDa protein in a genotype-specific manner in vivo, but not in vitro (Wolpert and Macko 1989). However, we recently found that native victorin binds to 100-, 65-, and 45-kDa proteins, as detected by Western blotting with anti-victorin antibody, in both susceptible and resistant oats, and both in vivo and in vitro (Akimitsu et al. 1992). These binding proteins, which bind victorin covalently, have been considered as possible candidates of victorin receptors (Wolpert and Macko 1989; Akimitsu et al. 1992). However, the relationship of the observed victorin binding to the specificity or toxicity of victorin is still unknown.

Anti-idiotypic antibodies raised against the idiotypes of antibodies to biologically important ligands have been used successfully to identify cell surface receptor sites in systems such as the insulin receptor (Sege and Peterson 1978), β-adrenergic receptor (Guillet et al. 1985; Schreiber et al. 1980), the nicotine receptor (Abood et al. 1987), the diphtheria toxin receptor (Roll et al. 1989), and other animal hormones, neurotransmitters, and lymphotropic viruses and factors (Strosberg 1989). Since the internal image of an anti-idiotypic antibody and the ligand epitope that binds to the receptor will be identical or very similar, the anti-idiotypic antibody also recognizes the ligand binding site of the receptor.

Anti-victorin antibody used in our victorin-binding studies could detect as little as 10 pg of victorin per milliliter in an indirect enzyme-linked immunosorbent assay (ELISA) (Akimitsu et al. 1992). In this study, we used this anti-victorin antibody as an immunogen to produce anti-victorin anti-idiotypic antibodies for further examination of victorin binding proteins.

RESULTS

Screening of anti-victorin anti-idiotypic antibodies.

Anti-victorin anti-idiotypic antibodies were assayed by the inhibition of victorin-HRP binding to anti-victorin antibody in a direct ELISA. When victorin was used as a competitor of victorin-HRP binding to anti-victorin antibody in the direct ELISA system, 25 ng of victorin per milliliter inhibited victorin-HRP binding by 50% and 1 μg of victorin per milliliter inhibited victorin-HRP binding by 97.5% (Akimitsu et al. 1992). When different concen-

Address correspondence to L. P. Hart.
Current address of K. Akimitsu: DOE-Plant Research Lab., MSU, East Lansing 48824 U.S.A.

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trations of protein A column-purified serum collected after
the third boost were mixed with victorin-HRP, as much as 84% of victorin-HRP binding was inhibited by the
addition of $1 \times 10^{-1}$ diluted serum and at least $4 \times 10^{-3}$
diluted serum still inhibited 33% of binding (Fig. 1). There
was no significant inhibition when preserum was used (Fig.
1). The increase of anti-idiotypic antibody production in
serum was correlated with the number of boosts (Fig. 2).

**Effects of anti-victorin anti-idiotypic
antibody on oat protoplasts.**

Victorin induces extracellular callose synthesis in sus-
cceptible oat protoplasts (Walton and Earle 1985). About
70% of susceptible oat protoplasts produced callose after
8 hr of incubation with 60 pg of victorin per milliliter,
callose production continued to increase for 24 hr
(Figs. 3A and 4). The secretion is host-specific, since no
induction occurred in resistant oat protoplasts after the
same treatment (Figs. 3B and 4). The 20-times diluted
serum (third boost) containing anti-victorin anti-idiotypic
antibodies induced callose synthesis in 10% of the sus-
cceptible oat protoplasts, but in none of the resistant oat
protoplasts (Figs. 3C and 5). The amount of callose pro-
duced from susceptible oat cells 18 hr after treatment with
anti-idiotypic antibodies was similar to that induced by
6 pg/ml of victorin (Fig. 3C,D). Treatment with a 1:20
dilution of preserum did not induce callose synthesis in
either susceptible or resistant oat protoplasts (Fig. 5). When
the same concentration of serum containing anti-idiotypic
antibodies was added simultaneously with 60 pg/ml of
victorin to oat protoplasts, inhibition of callose synthesis
was observed (Fig. 4). Callose synthesis was inhibited by
68% after 5 hr (Fig. 4). A 1:20 dilution of preserum had
no effect on the response to victorin (Fig. 4). Twenty
micrograms of BSA or human IgG per milliliter did not
induce callose and also had no effect on the callose secretion
induced by 60 pg/ml of victorin (Table 1).

**DISCUSSION**

Anti-victorin anti-idiotypic polyclonal antibodies were
produced in rabbits immunized with anti-victorin anti-
body-OVA conjugates. Since the antibody molecule is too
large to enter cells, anti-idiotypic antibodies are often used
for the determination of cell-surface receptors (Sega and
Peterson 1978; Strosberg 1989). Although a large number
of anti-idiotypic antibodies have been prepared (Strosberg
1989), this technique has rarely been used in a study of
plant-microorganism interactions.

In this study, we examined the effect of anti-victorin
anti-idiotypic antibodies on the production of callose from
cell. Extracellular synthesis of callose from susceptible
oat protoplasts is a very sensitive reaction of oat cells to
victorin (Walton and Earle 1985). Sixty picograms of
victorin per milliliter induced the synthesis of callose by
70% of the cells after 5 hr (Fig. 4) and as little as 6 pg
of victorin per milliliter stimulated callose secretion in some
cells (Fig. 3D) (Walton and Earle 1985). Although callose
synthesis is a sensitive response to victorin, there is no
evidence that this is the primary site of action of victorin.
Victron does not have an effect on glucan synthase II
callose synthase) activity in oat homogenates (Akimitsu
et al. 1992), and low concentrations of victorin also induce
other physiological responses, including ethylene (Shain
and Wheeler 1975) and avenalin synthesis (Mayama
et al. 1986).

The anti-idiotypic antibodies worked weakly as an
agonist on susceptible oat protoplasts (Fig. 5), but also
worked as an antagonist to the action of victorin (Fig.
4). These results may be explained by the polyclonal nature
of these anti-idiotypic antibodies which may consist of
several “internal images” of the binding part of the victorin
molecule to receptor. Several of the “internal images”
Fig. 3. The production of callose from oat protoplasts induced by victorin or by anti-victorin anti-idiotypic antibodies. Callose was stained with Calcofluor white and observed by fluorescence microscopy after 18 hr of incubation. All magnifications were 400X. A, Sensitive protoplasts treated with 60 pg/ml of victorin; B, resistant protoplasts treated with 60 pg/ml of victorin; C, sensitive protoplasts treated with 20-times diluted serum containing anti-victorin anti-idiotypic antibodies; and D, callose synthesis from sensitive protoplasts induced by 6 pg/ml of victorin.

Fig. 4. Inhibition of victorin-induced callose synthesis by anti-victorin anti-idiotypic antibodies. Twenty-times diluted anti-idiotypic serum or preserum was mixed with 60 pg/ml of victorin and treated to 0.5 ml of protoplast solution (4 x 10^6 protoplasts per milliliter). The number of cells making callose was counted and the percentage of total cells calculated. Error bars indicate the standard deviation of the mean of three repetitions. ●, Victorin, anti-idiotypic serum, susceptible protoplasts; ○, victorin, anti-idiotypic serum, resistant protoplasts; □, victorin, preserum, susceptible protoplasts; ▲, victorin, preserum, resistant protoplasts; △, victorin, susceptible protoplasts, no serum; Δ, victorin, resistant protoplasts, no serum.

Fig. 5. Induction of callose synthesis from oat protoplasts by anti-victorin anti-idiotypic antibodies. Twenty-times diluted anti-idiotypic serum or the same concentration of preserum was mixed with 0.5 ml of protoplast solution (4 x 10^6 protoplasts per milliliter). The number of cells making callose was counted and the percentage of total cells calculated. Error bars indicate standard deviation of the mean of three repetitions. ●, Anti-idiotypic antibodies, susceptible protoplasts; ○, anti-idiotypic antibodies, resistant protoplasts; □, preserum, susceptible protoplasts; ▲, preserum, resistant protoplasts; △, susceptible protoplasts, no serum (control); Δ, resistant protoplasts, no serum.
carried by the anti-idiotypic antibodies might lead to more than a single type of interaction with the receptor; some anti-idiotypic antibodies could act like victorin to induce callose synthesis, but other anti-idiotypic antibodies could block victorin binding to the receptor. Schreiber et al. (1980) reported that polyclonal anti-idiotypic antibodies against β-adrenergic worked as an agonist and also as an antagonist on ligand-receptor interactions (Schreiber et al. 1980). They speculated that multiple types of interactions of anti-idiotypic polyclonal antibodies to the β-adrenergic receptor occurred (Schreiber et al. 1980), and later found a monoclonal anti-idiotypic antibody which had only agonist properties of the physiological function of the original ligand (Guillet et al. 1985).

Our results using anti-victorin anti-idiotypic antibodies suggest that a victorin binding site related to victorin toxicity may exist on the surface of the plasma membrane of susceptible oat cells. The evidence supports the hypothesis that victorin receptor sites are on the plasma membrane of susceptible oats (Briggs et al. 1984; Bronson and Scheffer 1977; Gardner and Scheffer 1973; Haney and Wheeler 1968; Keck and Hodges 1973; Novacky and Haney 1974; Rancillac et al. 1976). The data suggest that anti-victorin anti-idiotypic antibody might be a useful tool to examine a cell surface receptor of victorin. However, the production of monoclonal anti-idiotypic antibody will probably be necessary for further examination of the receptor-anti-idiotypic antibody interactions.

MATERIALS AND METHODS

Plant materials.

Park was used as the C. victoriae-susceptible and victorin-sensitive oat cultivar, and Korwood was used as the C. victoriae-resistant and victorin-insensitive oat cultivar. Oats were grown 20–25 days in a growth chamber at 18°C under fluorescent and incandescent lamps (flux rate: 140 μmol/m/sec), with a 12-hr photoperiod.

Victorin C preparation.

The major form of victorin, victorin C (Wolpert et al. 1988), was isolated from 40 L of culture filtrates of C. victoriae (isolate 1146A) by previously described methods (Akimitsu et al. 1992; Mayama et al. 1986).

Victorin antibody production.

Anti-victorin antibodies were produced in rabbits immunized with victorin-bovine serum albumin (BSA) conjugate as described (Akimitsu et al. 1992). For the preparation of anti-victorin antibody-OVA conjugates, anti-victorin polyclonal antibodies were purified using immobilized-protein A and BSA columns (Akimitsu et al. 1992).

Production of anti-victorin anti-idiotypic antibody: immunogen preparation.

Anti-victorin antibodies purified by immobilized protein A and BSA columns were conjugated with ovalbumin in phosphate-buffered saline (PBS), pH 7.2, with glutaraldehyde (Langone and Bjercke 1989). Briefly, 1 mg of antibody was dissolved in PBS, pH 7.2, and mixed with 1 mg of ovalbumin. Eighty microliters of glutaraldehyde (25% commercial solution from Sigma) was added to the solution and incubated for 45 min at room temperature. The reaction was stopped by the addition of 200 μl of 2 M lysine, and the solution was dialyzed against four changes of PBS overnight at 4°C.

Immunization.

The antibody-OVA conjugate (0.5 mg) was dissolved in 1 ml of PBS and mixed with 1 ml of Freund’s complete adjuvant. The mixture was injected intradermally into three female New Zealand white rabbits, and boosted three times intramuscularly at 28-day intervals with 0.5 mg of conjugate in 1 ml of PBS plus 1 ml of Freund’s incomplete adjuvant. Blood was taken from rabbits 12 days after each injection, and serum was isolated (Harlow and Lane 1988).

The serum was purified by protein A column chromatography (Pierce/Immunopure IgG Purification kit), and the presence of anti-victorin anti-idiotypic antibodies was determined.

Screening for anti-victorin anti-idiotypic antibody.

The anti-victorin anti-idiotypic antibody was assayed by the inhibition of victorin-HRP binding to anti-victorin antibodies in a direct ELISA (Akimitsu et al. 1992; Strusberg 1989). Conjugation of victorin to HRP was described previously (Akimitsu et al. 1992). Twenty micrograms of anti-victorin antibody purified by both immobilized protein A and BSA columns in 100 μl of PBS (200 μg/ml) was placed in wells of a microtiter plate and incubated overnight at 4°C. The antibody solution was removed, and the well was washed with PBS-Tween 15 times. The plate was then incubated with 300 μl of PBS-Tween for 0.5 hr at 37°C to block unbound binding sites and washed with PBS-Tween five times. Victorin-HRP (diluted 1:10 from the stock solution [Akimitsu et al. 1992]) or victorin-HRP plus different concentrations of sample serum (purified by protein A column) or victorin-HRP plus different concentrations of victorin, in 1 ml of PBS-Tween, were placed on each well and incubated for 1 hr at 37°C. The plate was then washed 15 times with PBS-Tween, and 100 μl of HRP substrate (0.4 mM tetramethylbenzidine, 0.004% H₂O₂, in 50 mM sodium acetate, pH 6.0) was added. After 30 min of incubation at room temperature, the reaction was stopped by addition of 2 M H₂SO₄, and absorbance at 450 nm was read with a Bio-Tec ELISA reader.

Table 1. Effect of proteins on victorin-mediated callose synthesis

<table>
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<tr>
<th>Treatment</th>
<th>Cells producing callose (%)</th>
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<tbody>
<tr>
<td>Victorin (60 μg/ml)</td>
<td>68.9 ± 5.9</td>
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<tr>
<td>Victorin (60 μg/ml) plus BSA (20 μg/ml)</td>
<td>75.5 ± 6.7</td>
</tr>
<tr>
<td>Victorin (60 μg/ml) plus human IgG (20 μg/ml)</td>
<td>69.8 ± 3.5</td>
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*Concentrations listed are the final concentrations in 0.5 ml of oat protoplast solution (4 × 10⁶ protoplasts per milliliter).
Protoplast preparation.
Sterile protoplasts were prepared by the methods described by Schaeffer and Walton (1990). Green oat leaves (500 mg) were sterilized with 10% bleach, abraded with Carborundum, and incubated at 26°C for 3 hr in 2% (w/v) Cellulysin in SCM buffer (0.5 M sorbitol, 10 mM CaCl₂, and 40 mM Mes, pH 5.5) with gentle shaking. Released protoplasts were filtered through 80-µm nylon screen mesh, washed twice with SCM (pH 5.8), and collected by centrifugation at 100 × g for 5 min. The protoplasts were washed with SCM (pH 5.8) by centrifugation two more times, and resuspended in SCM (pH 5.8) at a final concentration of 4 × 10⁴ protoplasts per milliliter.

Anti-vitoxic anti-idiotypic antibody treatment of oat protoplasts.
Protoplasts (0.5 ml of 4 × 10⁴ protoplasts per milliliter) were placed in wells of 24-well Falcon multiwell plates (Becton-Dickinson), and mixed with vitoxic (60 pg/ml) and/or 1:20 diluted serum containing anti-idiotypic antibodies. BSA, human IgG (protein standard from Sigma), and preserum, were used as controls. The protoplasts were incubated in the dark for various periods at room temperature, and callose production was observed by staining with a 1:1 mixture of 0.1% Calcofluor white and SCM (pH 5.8) and viewing under a Zeiss epifluorescence microscope (filter set G365) at a final magnification of 400X.

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LITERATURE CITED