Host-Pathogen Interactions

XXXII. A Fungal Glucan Preparation Protects Nicotiana against Infection by Viruses

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ABSTRACT

A glucan preparation obtained from the mycelial walls of the fungus Phytophthora megasperma f.sp. glycinea and known as an elicitor of phytoalexins in soybean was shown to be a very efficient inducer of resistance against viruses in tobacco. The glucan preparation protected against mechanically transmitted viral infections on the upper and lower leaf surfaces. Whether the glucan preparation was applied by injection, inoculation, or spraying, it protected the plants if applied before, at the same time as, or not later than 8 hours after virus inoculation. At concentrations ranging from 0.1 to 10 micrograms per milliliter, the glucan preparation induced protection ranging from 50 to 100% against both symptom production (necrotic local lesions, necrotic rings, or systemic mosaic) and virus accumulation in all Nicotiana-virus combinations examined. However, no significant protection against some of the same viruses was observed in bean or turnip. The host plants successfully protected included N. tabacum (9 different cultivars), N. sylvestris, N. glutinosa, and N. clevelandii. The viruses belonged to several taxonomic groups including tobacco mosaic virus, alfalfa mosaic virus, and tomato black ring virus. The glucan preparation did not act directly on the virus and did not interfere with virus disassembly; rather, it appeared to induce changes in the host plant that prevented infections from being initiated or recently established infections from enlarging. The induced resistance does not depend on induction of pathogenesis-related proteins, the phenylpropanoid pathway, lignin-like substances, or cellulose-like materials. We believe the induced resistance results from a mechanism that has yet to be described.

The hypersensitive reaction of plants to viruses is characterized by the appearance of necrotic local lesions with the virus localized within the lesions and surrounding areas. The living cells surrounding the lesions undergo marked metabolic changes that are believed to cause, or at least to contribute to, the observed resistance (for recent reviews, see 8, 26). The metabolic alterations that occur during a viral-induced hypersensitive reaction appear to be similar to those that occur in the same host during incompatible interactions with pathogenic bacteria and fungi (7). The most commonly observed metabolic changes are the production of phytoalexins and phenylpropanoid metabolites. In previous reports, it was shown that the phenylpropanoid pathway is strongly activated (15) in tobacco leaves reacting hypersensitively to TMV and that the virus localizing mechanism was weakened (larger lesions) if infected leaves were treated with competitive inhibitors of phenylalanine ammonia-lyase, a key enzyme in the pathway (19, 20). This suggested an involvement of the activated phenylpropanoid pathway in the localizing mechanism.

Treatment of various plant materials with elicitors of microbial origin have been shown to mimic defense responses and to stimulate the pathways leading to phenylpropanoids and flavonoids (for reviews, see 1, 4). Among these elicitors were cell wall fragments and substances present in the culture filtrates of the fungus Phytophthora megasperma f.sp. glycinea (Pmg) (1, 2). In particular, poly- and oligosaccharides extracted and purified from cell walls of this fungus were shown to exhibit very high phytoalexin elicitor activity in soybean cotyledons. These elicitors have been identified as glucans characterized by the presence of terminal 3-, 6-, and 3,6-linked glucosyl residues linked in the β configuration (1, 2, 5). We used a preparation of Pmg glucan in an attempt to pre stimulate defense responses in tobacco leaves and then examined its effect on the normal hypersensitive response induced by TMV. This approach led to the surprising observation that treatment of tobacco leaves with the Pmg glucan preparation inhibited dramatically the production of virus-induced necrotic local lesions. The results described herein summarize the effect, on viral infections, of treating tobacco plants with the glucan preparation. We show that the glucan preparation efficiently protected the plants against viral infection without directly affecting the virus structure in such a way as to suppress infectivity. The glucan preparation-induced

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2Abbreviations: TMV, tobacco mosaic virus; Pmg, Phytophthora megasperma f.sp. glycinea; AVM, alfalfa mosaic virus; TBRV, tomato black ring virus; PAL, phenylalanine ammonia lyase; OMT, O-methyl transferase; AOPP, α-aminoxy-β-phenyl-propionic acid; PR proteins, pathogenesis-related proteins.
protection was host-mediated and was observed for all of the combinations of *Nicotianae* and viruses so far tested.

**MATERIALS AND METHODS**

**Pmg Glucan Preparation**

Mycelia were collected and homogenized, the mycelial walls purified and partially hydrolyzed with acid, and the void glucan purified as described (28). In summary, lyophilized Pmg mycelial walls were suspended (1 g/100 mL) in 2 N-trifluoroacetic acid, and the container was placed in an 85°C water bath for 2.5 h, with shaking at 30 min intervals. Insoluble residues were then removed by centrifugation followed by passage through a GF/C filter. Residual TFA was removed by retroevaporation at 35 to 40°C under reduced pressure. The resulting syrup was dissolved in 10 mL of water, neutralized with 1 N NaOH and brought to 30 mL. The material that eluted in the void volume of a Bio-Gel P-2 gel permeation column was the glucan preparation used for the experiments described in this paper (28).

**Plants**

The host plants used in most experiments were *Nicotiana* species, namely *N. tabacum* (cvs Samsun, Samsun NN, Samsun H, Xanthi nc, White Burley, Coulou, P19, Corolle XX, and Judy's Pride), *N. sylvestris*, *N. glutinosa*, and *N. clevelandii*. *Phaseolus vulgaris* (cvs Saxa and Pinto) and *Brassica rapa* L. cv Just Right were also used in a few experiments. The plants were grown in a greenhouse under controlled conditions. The plants were placed in a growth chamber at 22 ± 1°C (16 h of photoperiod, 2 × 10^2 W m^-2 total irradiance), several days before and during the incubation period following inoculation with virus and treatment with glucan.

**Viruses**

TMV strains were purified from infected tobacco leaves as described (33). The S-strain of alfalfa mosaic virus (AIMV), the S-strain of tomato black ring virus (TBRV), and the Cabb-S strain of cauliflower mosaic virus were gifts of Dr. C. Stussi-Garaud, Dr. C. Fritsch, and Prof. G. Lebeurier, respectively, from the Strasbourg Institute. ^3H-Labeled TMV was prepared as described (14).

**Inoculation of Leaves with Virus**

Virus infection was usually accomplished by inoculation of the two or three first fully expanded leaves by rubbing the upper surface with a glass pad and abrasive Celite in the presence of a suspension containing purified virus. In some experiments, inoculation was similarly performed on the lower surface of the leaves. Virus concentrations were adjusted in order to induce about 200 local lesions or necrotic rings per control leaf (not treated with glucan).

**Treatment of the Leaves with the Glucan Preparation**

In most of the experiments, the glucan preparation was mixed with virus and applied by inoculation. In some experiments, the glucan preparation was applied by spray or by injection, using a syringe with a very fine needle inserted in the leaf tissue in the vicinity of lateral veins.

**Measurements of Virus Amounts**

TMV and AIMV were quantitated with an indirect ELISA (32). The leaf samples to be assayed were ground in a mortar in the presence of quartz sand and in 5 mL/g fresh weight of PBS-Tween buffer (0.02 M phosphate, 0.15 M NaCl, 0.003 M KCl [pH 7.4], containing 0.05% Tween-20). The homogenate was centrifuged at 10,000g for 30 min and the supernatant used as such or serially diluted with PBS-Tween buffer for the antigen incubation step of the ELISA procedure. The conditions of incubation and the washing steps have already been described (14, 32). The specific steps of the procedure included adding to the wells: (a) hen (25) anti-TMV or anti-AIMV immunoglobulins (coating antibody, 50 μg/mL in 0.05 M sodium carbonate [pH 9.6]), (b) 1% bovine serum albumin in PBS-Tween, (c) antigen in the same buffer (standard solutions 0–1 μg/mL of purified TMV or AIMV or crude extracts of infected leaf tissue), (d) rabbit anti-TMV or anti-AIMV immunoglobulins (10 μg/mL, diluted in PBS-Tween buffer), and, finally, (e) goat anti-rabbit immunoglobulins conjugated with alkaline phosphatase (Sigma, 1 μg/mL in PBS-Tween buffer). The bound enzyme conjugate was measured as described (14, 32). The lower limit of virus detection and quantitation was 5 ng/g fresh weight.

**RESULTS**

**Effects of Treatment with the Glucan Preparation on Virus-Induced Local Lesion Formation**

The tobacco cultivar Samsun NN is a useful test host for TMV because the number and location of the infection sites are easily visualized as local necrotic lesions (surrounded by rings exhibiting bright blue fluorescence in ultraviolet light) and because the extent of virus multiplication at each infection site is reflected by the size of the lesion. TMV-induced local lesions appear 33 to 36 h after virus inoculation of tobacco leaves. In our first experiments, in which the aim was to modulate the hypersensitive response, the glucan preparation was injected into the mesophyll tissue at least 12 h before the normal time of appearance of the local lesions. We did not observe the predicted effect (smaller lesions) of treatment with the glucan preparation. The size of the lesions that developed on treated leaves was not significantly different from those on control leaves, whatever the timing of application of the glucan preparation and virus. Unexpectedly, there was a dramatic reduction in the number of lesions when the glucan preparation was applied shortly after, at the same time as, or as much as 25 d before virus inoculation. Inhibition of lesion formation was also observed when the glucan preparation was sprayed on the leaves prior to virus inoculation or when the glucan preparation and virus were mixed and inoculated together (Fig. 1). Inoculation was used in most of our later experiments, since it enabled an accurate determination of the concentration and amount of both the virus and the glucan preparation applied to the leaves.

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Determining Whether the Virus is Inactivated by or Otherwise Associated with the Glucan Preparation

Samsun NN leaves were inoculated with 0.2 μg/mL TMV and 10 μg/mL of the glucan preparation, i.e., conditions that lead to the situation illustrated by Figure 1D. Ten d later, the amount of virus in the symptom-free leaves was determined by ELISA. The virus titers were background levels; this result provided evidence that the glucan preparation did not simply inhibit the necrotization process in Samsun NN without interfering with virus infection. Rather, the glucan preparation treatment appeared to fall into the class of viral inhibitors that prevents infection from being initiated (3, 10). Such inhibitors could act by inactivating the virus, for instance by binding to it and forming a complex that would suppress infectivity. This possibility was investigated by gel-permeation chromatography, using highly purified 3H-labeled TMV and an unlabeled glucan preparation.

3H-Labeled TMV (100 μg) and the glucan preparation (200 μg) were mixed in 1 mL (the same ratio that yielded a high degree of protection [Figs. 1 and 3]), and the relatively concentrated mixture was subjected to gel-permeation chromatography on Ultrogel AcA34 (Fig. 2B). As a control, 3H-labeled TMV without the glucan preparation was chromatographed (Fig. 2A). The 3H label allowed a more sensitive detection of the viral RNA than the absorbance at 260 nm. TMV by itself had the same elution profile as TMV that had been mixed with the glucan preparation (Fig. 2). The amount of TMV recovered was the same in both cases, as shown by the areas of the absorbance and radioactivity peaks. The infective TMV particles were fully recovered from the mixture; the infectivity peak was superimposable on the absorbance and radioactivity peaks (Fig. 2). Furthermore, the glucan preparation, which eluted as a broad peak later in the column (Fig. 2), retained its full ability to protect tobacco plants from viral infection. These results demonstrated that the protective effects of the glucan preparation were not due to direct inactivation of the virus nor to the binding of glucan or other components in the glucan preparation to the virus in a manner that could have prevented the stripping of the virus coat protein and thereby reduced infectivity. We concluded that the effect of the glucan preparation was on the host plant rather than the virus.

Effects of the Glucan Preparation on Symptoms Produced by Several Viruses in Various Plant Species

The very low level of TMV found in glucan preparation-protected leaves of Samsun NN indicated that, at each potential infection site created by the mechanical inoculation, there
was very little or no virus multiplication. This suggested that the glucan preparation acts early in the process of TMV infection in Samsun NN, and that the glucan preparation might protect Samsun NN from infection by other viruses. The results of experiments to examine this question showed that the glucan preparation not only protected Samsun NN from TMV, but protected all twelve Nicotiana species and cultivars tested from TMV, TMV-RNA, AIMV, and TBRV (Table I). In contrast, the glucan preparation did not protect other plant species tested from viral infection (Table I). This observation provided additional evidence that the glucan preparation did not directly interfere with the viruses, but rather interacted with the Nicotiana plants.

It is also noteworthy (Table I) that the glucan preparation protected Nicotiana against infection by naked viral RNA. Therefore, the mechanism by which the glucan preparation protects the plants cannot be interference with disassembly of the virus, an early event in plant virus infection.

**Glucan Preparation-mediated Inhibition of Symptom Development and of Virus Accumulation as Dose-Dependent Effects**

Infection sites that develop successfully can be visualized for several of the plant-virus combinations listed in Table I. In such cases, the number of infection sites was estimated 2 to 4 d after inoculation. This was particularly true for combinations yielding well-defined necrotic local lesions, such as tobacco Samsun NN-TMV (virus particles or naked viral RNA), tobacco Xanthi nc-TMV, tobacco Samsun H-TMV, *N. glutinosa*-TMV and *N. sylvestris*-U2 strain of TMV. For TMV, the results demonstrated that glucans produced by *Samsun* cv NN was easy to count after inoculation with TBRV or AIMV. However, when the combinations yielded systemic mosaic (for instance, *N. tabacam* cvs Samsun, White Burley, Coulou, P19, Corolle XX, Judy's Pride, and *N. sylvestris* infected with the U1 strain of TMV), the symptoms were visible only about 1 week after inoculation, and no fluorescence was detectable.

The effect of varying the glucan preparation concentration on production of necrotic lesions in various host-virus combinations is illustrated in Figure 3. A. For concentrations of 1–10 μg/mL, the glucan preparation inhibited the production of symptoms, for the various combinations examined, in a reproducible manner. Lower concentrations of the glucan preparation resulted in more variable degrees of protection. The variability was primarily the result of differences in susceptibility to infection exhibited by one batch of plants as compared to another, as well as the variability introduced by plants grown at different periods of the year. These variables are known to cause the number of local lesions or necrotic spots induced by a given number of viral particles to vary over one order of magnitude (21). However, when the control leaves of a particular group of plants exhibited a 10-fold higher susceptibility to virus infection than another group of plants, the concentration of the glucan preparation required for efficient protection had to be increased only 2- to 3-fold. Thus, the effect of the glucan preparation was more reproducible than the susceptibility of the host to mechanical abrasion and virus infection.

Three host-virus combinations yielding necrotic local lesions, necrotic spots, or systemic mosaic symptoms (Table I) were tested to determine the ability of the glucan preparation to inhibit virus production in the inoculated leaves as measured by ELISA (Fig. 3B). The effect of the glucan preparation on virus production in these combinations was very similar to the effect on the number of lesions in these instances where the virus infection resulted in necrotic local lesions (cf. Fig. 3,
A and B). Since about 200 μL of liquid containing both the glucan preparation and virus was necessary for inoculation of one leaf, it was concluded (from Fig. 3) that 20 ng glucan per leaf induced significant protection and that 0.2 to 1 μg glucan per leaf completely prevented infection and symptoms from occurring.

The ability of the glucan preparation applied with the virus to provide protection against systemic infection is illustrated for the *N. sylvestris*-TMV-U1 combination in Figure 4. *N. sylvestris* exhibits pronounced disease symptoms several weeks after virus inoculation (Fig. 4C). When the glucan preparation was added at 10 μg/mL to the virus inoculum, *N. sylvestris* was protected completely from disease. It is noteworthy that the glucan caused no phytotoxic symptoms (Fig. 4, A and B).

**Effect of the Glucan Preparation on the Escape of Infectious Material from an Inoculated Leaf and on Its Systemic Spread in the Plant**

TMV, in a systemic host such as *N. tabacum* cv Samsun, multiplies in the inoculated leaf, after which the infection spreads to the tip of the plant and then progressively to the leaves situated below the tip (21) (see also Fig. 5, at 0 concentration of the glucan preparation). After infection, virus amounts reach a maximum and then decrease in all parts of the plant. About 1 week after inoculation, mosaic symptoms appear and are most pronounced on the leaflets of the plant tip and on the leaves situated just below the leaflets, where the virus multiplies at high rates. When the glucan preparation had been added at 10 μg/mL to the inoculum, no mosaic or any other symptoms were detectable on the Samsun plants.

The absence of symptoms was correlated with the absence of detectable virus in all parts of the plant (Fig. 5). In plants that had been treated with smaller amounts of the glucan preparation (0.1 μg/mL), appearance of mosaic symptoms was delayed, and the severity of the symptoms was less after 18 d than in untreated, infected plants after 11 d. Furthermore, the protection conferred by 0.1 μg/mL of the glucan preparation in the inoculum, though incomplete, was responsible for a delay in the spreading of the virus to the plant tip and

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### Table I. Effects of the Glucan Preparation on Symptoms Produced by Several Viruses in Various Plant Species

<table>
<thead>
<tr>
<th>Plant Species (cv)</th>
<th>Virus</th>
<th>Normal Symptoms</th>
<th>Effects of Glucan</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>N. tabacum</em> (Samsun NN)</td>
<td>TMV-U1</td>
<td>Local lesions</td>
<td>Reduction in number</td>
</tr>
<tr>
<td></td>
<td>TMV-U1-RNA</td>
<td>Local lesions</td>
<td>Reduction in number</td>
</tr>
<tr>
<td></td>
<td>A1MV</td>
<td>Necrotic spots</td>
<td>Reduction in number</td>
</tr>
<tr>
<td></td>
<td>TBRV</td>
<td>Necrotic spots</td>
<td>Reduction in number</td>
</tr>
<tr>
<td>(Xanthi nc)</td>
<td>TMV-U1</td>
<td>Local lesions</td>
<td>Reduction in number</td>
</tr>
<tr>
<td>(Samsun H)</td>
<td>TMV-U1</td>
<td>Local lesions</td>
<td>Reduction in number</td>
</tr>
<tr>
<td>(Samsun)</td>
<td>A1MV</td>
<td>Necrotic spots</td>
<td>Reduction in number</td>
</tr>
<tr>
<td></td>
<td>TBRV</td>
<td>Necrotic spots</td>
<td>Reduction in number</td>
</tr>
<tr>
<td>(White Burley)</td>
<td>TMV-U1</td>
<td>Systemic mosaic</td>
<td>Delay or suppression</td>
</tr>
<tr>
<td>(Coulro)</td>
<td>TMV-U1</td>
<td>Systemic mosaic</td>
<td>Delay or suppression</td>
</tr>
<tr>
<td>(P19)</td>
<td>TMV-U1</td>
<td>Systemic mosaic</td>
<td>Delay or suppression</td>
</tr>
<tr>
<td>(Corolle XX)</td>
<td>TMV-U1</td>
<td>Systemic mosaic</td>
<td>Delay or suppression</td>
</tr>
<tr>
<td>(Judy's Pride)</td>
<td>TMV-U1</td>
<td>Systemic mosaic</td>
<td>Delay or suppression</td>
</tr>
<tr>
<td><em>N. glutinosa</em></td>
<td>TMV-U1</td>
<td>Local lesions</td>
<td>Reduction in number</td>
</tr>
<tr>
<td><em>N. sylvestris</em></td>
<td>TMV-U1</td>
<td>Systemic mosaic</td>
<td>Delay or suppression</td>
</tr>
<tr>
<td></td>
<td>TMV-U2</td>
<td>Necrotic spots</td>
<td>Reduction in number</td>
</tr>
<tr>
<td><em>N. clevelandii</em></td>
<td>TBRV</td>
<td>Necrotic spots</td>
<td>Reduction in number</td>
</tr>
<tr>
<td>Phaseolus vulgaris</td>
<td>(Saxa)</td>
<td>A1MV</td>
<td>Local lesions</td>
</tr>
<tr>
<td></td>
<td>(Pinto)</td>
<td>TMV-U1</td>
<td>Local lesions</td>
</tr>
<tr>
<td>Brassica rapa L.</td>
<td><em>Cauliflower</em></td>
<td>Mosaic virus</td>
<td>Systemic mosaic</td>
</tr>
<tr>
<td></td>
<td>(Just Right)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Symptoms of virus infection could be divided into three groups: local necrotic lesions surrounded by well delimited fluorescent rings, necrotic spots surrounded by broad zones of fluorescence, and systemic mosaic without detectable fluorescence.

* Effect of the glucan preparation was either a reduction in number of local lesions or of necrotic spots or a delay or suppression of mosaic symptoms; in some hosts the effects were not significant or were not reproducible.
to the fourth leaf (Fig. 5). We concluded that, in systemic hosts, the glucan preparation efficiently reduced the number of initiated infection sites on the inoculated leaves, and this in turn reduced the spread of the infection from the inoculated to the uninoculated leaves in which virus accumulation and symptom development were then considerably delayed.

Relationship between the Order of Application of Virus and the Glucan Preparation and the Efficiency of Protection

The results obtained with systemically as well as hypersensitively reacting hosts suggested that the glucan preparation interfered at an early stage of infection or prevented the normal establishment of infection sites. This question was further investigated by varying the order and time of application of virus and the glucan preparation. Maximum protection was observed when the glucan preparation was applied (by injection, inoculation, or spray) up to 25 d before or at the same time as virus inoculation (Fig. 6). The protection efficiency of the glucan preparation at 10 μg/mL dropped from 100% to about 80% when applied 1 h after virus inoculation (Fig. 6). Surprisingly, after 1 h, the rate of decreasing protection slowed down so that the glucan preparation still afforded about 80% protection when applied 8 to 10 h after virus inoculation. However, the glucan preparation, at 10 μg/mL, lost all apparent ability to protect the plant when applied 12 to 24 h after virus inoculation. Similar results were obtained when the glucan preparation was injected instead of inoculated. These results demonstrated that the glucan preparation induced changes in the host that prevented infections from being initiated as well as preventing recently established infections from enlarging.

Mechanism by Which the Glucan Preparation Protects Tobacco Plants against Virus Infection Is Apparently Not a Known Stress/Defense Response

One of the commonly observed responses in tobacco exhibiting hypersensitive resistance to virus infection is a very large induction of PAL and OMT. PAL is the first enzyme in the phenylpropanoid pathway, while OMT activity is accounted for by three isoforms, each of which is able to catalyze two later steps in the pathway, i.e. methylation of caffeic acid to ferulic acid and of 5-hydroxyferulic acid to sinapic acid (16). Treatment of tobacco with the glucan preparation resulted in a very slight increase in the activities of these enzymes. However, these increases were much lower than those previously observed (15) during the hypersensitive response to
Figure 6. Effect on the protection of the order of application of virus and the glucan preparation, and of the interval of time between applying the virus and the glucan preparation, or between applying the glucan preparation and the virus. (●), 400 μL of a solution of 10 μg/mL of the glucan preparation or of water were applied by spray on the four fully developed leaves of N. tabacum Samsun NN plants; at various intervals (in days) after glucan preparation application, treated leaves were inoculated with 200 μL of 0.2 μg/mL TMV. Protection in percent was calculated, from the the number of lesions on glucan preparation-treated leaves (nt) and on the corresponding water-treated leaves (nc), as 100 (1-nt/nc); (nt) and (nc) are the means calculated from 12 glucan preparation-treated and 12 water-treated leaves, respectively. (○), Three fully developed leaves of N. tabacum Samsun NN plants were first inoculated with 200 μL of 0.2 μg/mL TMV; at various intervals (in hours) after virus inoculation, one-half of every leaf was inoculated with 100 μL of 10 μg/mL of the glucan preparation and the other half was inoculated with 100 μL water. Protection in percent was again calculated from the number of lesions on glucan preparation-treated leaf halves (nt) and on the corresponding water-treated halves (nc) as 100 (1-nt/nc). The values are the means calculated from two replicate experiments; for each determination, three leaves of three plants were used.

TMV infection, especially if the stimulated activities are expressed on a per cell basis, i.e. if the results are corrected by taking into account the number of cells affected by the changes. When the glucan was injected into the mesophyll cells, PAL and OMT were increased in many cells to a level that was 1.5-fold over the water-injected control. In leaves reacting hypersensitively and carrying local lesions, PAL and OMT increased 10- to 20-fold over the control (18), but the increase was restricted to the rings of cells surrounding the lesions. Although the effect of the glucan preparation on PAL and OMT activities was apparently too small to explain the induced resistance to virus, we decided to determine what would happen to the ability of the glucan preparation to protect against viral infection if the formation of lignin was selectively inhibited with AOPP (20).

Treatment of Samsun NN leaves with 100 μM AOPP and TMV resulted in lesions 40% larger than in similar leaves not treated with AOPP. Thus, the AOPP apparently interfered with the hypersensitive resistance response of the plant. Treatment of similar leaves with TMV and the glucan preparation (10 μg/mL) resulted in 99% protection, regardless of whether 100 μM AOPP was included with the treatment. Thus, inhibition of lignin synthesis does not interfere with the glucan preparation's ability to induce the leaves to protect themselves from viral infection.

The response of Solanaceae to environmental, chemical, pathogenic, or even hormonal stress is accompanied by accumulation of the well-known PR proteins (for a review, see ref. 31). Four PR proteins in tobacco have recently been shown to be 1,3-β-glucanases, and four other PR proteins were identified as chitinases (13, 17). Experiments were performed to see if the glucan preparation (10 μg/mL) induced PR proteins in Samsun NN tobacco leaves by attempting to detect the enzymic activities of the PR proteins and by looking for the PR proteins on native polyacrylamide electrophoresis slab gels. By these criteria, the glucans did not induce any of these PR proteins (data not shown).

The Samsun NN leaves treated with 10 μg/mL of the glucan preparation were examined microscopically. There was no apparent injury to the leaf cells and no apparent increase in aniline blue positive material (callose) or phloroglucinol positive material (lignin).

These results indicate that the Pmg glucan preparation probably does not protect plants by a known defense response or by any stress known to induce PR proteins (12).

**DISCUSSION**

Inhibitors of plant viruses may be divided into two categories (3, 10): those that inhibit virus infection and those that inhibit virus multiplication. The former are substances that inactivate virus infectivity in vitro or that, when applied to leaves simultaneously with virus inoculation, prevent infection from occurring. The latter are substances that, when applied to leaves already infected, retard the rate at which the virus replicates and spreads. The results presented above demonstrated that the Pmg glucan preparation falls into the first category, i.e. it is a viral inhibitor that interferes at early stages before and/or after initiation of the infection.

Inhibitors of viral infection have been studied more extensively than have inhibitors of viral multiplication, but their mode of action has not been elucidated. Some inhibitors of viral infection have been found in extracts of plants and appear to be proteinaceous in nature (3, 10). Other inhibitors of viral infection have been obtained from bacteria, yeast, and fungi (for reviews, see refs. 3, 10). It was suggested as long ago as the 1930s that some inhibitors of viral infection might be polysaccharides, because the inhibitors tolerated boiling and high concentrations of ethanol. But only in two cases have such polysaccharides been partially purified and even minimally characterized. Furthermore, in those cases, even after purification, rather high concentrations (100–1000 times the concentration used in the present studies) had to be applied to the plants for efficient protection (9, 29).

We report here on the high antiviral activity of a glucan preparation from Pmg cell walls, a preparation that had previously been shown to be an active elicitor of phytoalexin accumulation in soybean cotyledons (1, 2, 5). In the present study, the glucan preparation protected Nicotiana from virus particularly well when the glucan preparation was applied before or at the same time that the plant was inoculated with.
the virus. The glucan preparation was active if applied by injection, inoculation, or spray. The plants were protected regardless of whether the virus inoculation and the glucan preparation treatment were carried out on the upper or lower surface of the leaves. A remarkable reproducible protection was observed for a given concentration of the glucan preparation with all the Nicotiana-virus combinations examined, even though different concentrations of different viruses were necessary to establish approximately the same number of infection sites on various Nicotiana species (Table I; Fig. 3). Whereas a high degree of the glucan preparation-mediated protection was observed in Nicotianae against TMV and AYMV, no significant protection against these two viruses was obtained after treatment of bean with the glucan preparation.

The above results and the ability to recover fully infectious TMV, after mixing with and subsequent separation from the glucan preparation, showed that the glucan preparation was acting on the host and not on the virus. This contrasted with a report (22) in which electron microscope studies revealed a 'coating' of TMV with an inhibitor of virus infection that was a carbohydrate obtained from Physarum polycephalum. In that study, the binding of the carbohydrate to the virus could have inhibited stripping of the virus particle and, therefore, the subsequent steps necessary for infection. In our results, the glucan preparation protected equally well against infection by naked viral RNA and viral particles, thereby showing that inhibition of disassembly was not involved in the mode of action of the glucan preparation.

There are also marked differences between the results of our experiments and those reported previously on the protection of various Nicotianae against several viruses by a polysaccharide fraction isolated from Phytophthora infestans (11, 29). The P. infestans polysaccharide was characterized as a water-soluble β(1,3)-linked-d-glucan with an average degree of polymerization of 23 glucose units and with a single branching point (35). The Pmg polysaccharide preparation used in the present study was largely composed of a highly branched β-D-glucan with terminal, 3-, 6-, and 3,6-linked glucosyl residues (28). When coinoculated on Nicotianae with various viruses, variable concentrations of the P. infestans polysaccharide, ranging from 500 to 5000 µg/mL, were necessary to protect against formation of local lesions, except in the case of the tobacco Samsun-potato virus × combination where 100 µg/mL was effective. In our experiments, almost full protection was achieved with concentrations of glucan that were 2 to 3 orders of magnitude lower, and the efficiency of protection was independent of the Nicotiana-virus combination examined.

Another striking difference between the modes of the action of the P. infestans and Pmg glucan preparation is concerned with the effects on virus production. It was found (29) that the P. infestans polysaccharide preparation completely inhibited local lesion development without inhibiting virus multiplication. In contrast, we found that the Pmg glucan preparation inhibited symptom production and virus production in parallel (Fig. 3); a very sensitive ELISA procedure did not detect, even several weeks after virus inoculation, any virus in Pmg-glucan-preparation-treated symptomless tissues (Figs. 1D and 4B). This provided evidence that virus multiplication was inhibited during or soon after the establishment of infection sites. Even when the Pmg glucan preparation was applied several hours after virus inoculation, the glucan preparation was still capable of preventing the development of about 80% of the infection sites (Fig. 6).

It is generally assumed that infection spreads from the first infected epidermal cell(s) to neighboring cells 1 to 2 h after virus inoculation, and from epidermal cells to mesophyll cells 5 to 10 h after inoculation (6, 34). Thus, the epidermal layer is the most likely primary site of action of the glucan preparation (Fig. 6). The results summarized in Figure 6 suggest that the glucan preparation can no longer restrict the infection once the virus has spread to mesophyll cells.

It is interesting to compare the conditions required for efficient protection of plants by spraying with the glucan preparation with those reported recently for transgenic plants expressing the viral coat protein gene of TMV (23, 24, 27, 30). In the latter case, protection was first detected in systemically reacting hosts by a delay in symptom appearance that could be overcome by increasing virus in the inoculum (27). More recently, it was reported that, in the transgenic plants, the protection resulted from a reduction in the number of successful infection sites (23), which is also true of glucan preparation-mediated protection. In both systems, a reduction in the number of initial infection sites amplified the delay in symptom expression and in virus production.

The mechanism by which the glucan preparation causes the tobacco plants to resist virus infection remains an intriguing mystery. The glucan preparation-induced biochemical changes associated with anti-viral activity in tobacco are now under investigation.

**LITERATURE CITED**


