Use of Monoclonal Antibodies in the Purification of an Inhibitor of Virus Replication by Affinity Chromatography

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SUMMARY

Mouse monoclonal antibodies (MAbs) were prepared to an inhibitor of virus replication (IVR), released from protoplasts or leaf tissue of hypersensitive tobacco plants infected with tobacco mosaic virus. The MAbs were highly specific for IVR and reduced its antiviral activity. Using these MAbs in affinity chromatography enabled the recovery of purified IVR. SDS-PAGE of the immunoaffinity-purified IVR gave a single Mr 23K band. Immunoblots of IVR from extracts of protoplast or leaf tissue also revealed a single Mr 23K band which suggests that protoplast and tissue IVR are closely related.

In our previous studies we reported that a substance(s) inhibiting virus replication is released into the medium from tobacco mosaic virus (TMV)-infected protoplasts of a hypersensitive tobacco cultivar. The inhibitor of virus replication (IVR) from protoplasts was partially purified using zinc acetate precipitation (crude protoplast IVR) and two biologically active proteins with Mr of approx. 26K and 57K (fractionated protoplast IVR-1 and IVR-2 respectively) were obtained by gel filtration (Loebenstein & Gera, 1981). Recently, IVR was also obtained from the intracellular fluid of hypersensitive tobacco leaves infected with TMV (tissue IVR) (Spiegel et al., 1989). A distinct band (23K) that was not present in control preparations was regularly observed after PAGE of crude protoplast and tissue IVR. Electroelution of the 23K protein band from the gel yielded a biologically active preparation of IVR (A. Gera, G. Loebenstein, A. Frank & S. Harlap, unpublished results). When polyclonal antisera prepared against fractionated IVR-1, IVR-2 and a mixture of IVR-1 and IVR-2 (Gera & Loebenstein, 1989), were used in immunoblot analysis of crude protoplast IVR at least two bands reacted (A. Gera et al., unpublished results).

Here we report the preparation of monoclonal antibodies to protoplast IVR and their use for further purification of IVR by affinity chromatography and for detecting IVR from protoplast and tissue extracts in immunoblots.

Crude protoplast IVR and fractionated protoplast IVR-1 were prepared as described previously (Loebenstein & Gera, 1981). Tissue IVR was obtained from the intercellular fluid of Nicotiana tabacum L. cv. Samsun NN inoculated with TMV. A preparation obtained from 1 g leaf tissue will be termed one ‘unit’. This was found to be roughly equivalent to the amount of IVR obtained from 10⁶ protoplasts. One unit of IVR is equivalent to approx. 10 ng protein (Spiegel et al., 1989). Crude protoplast IVR was further purified by immunoaffinity chromatography, using purified immunoglobulin G from IVR polyclonal serum coupled to CNBr-activated Sepharose 4B (Pharmacia) as described by the manufacturer. Fifty units of lyophilized crude protoplast IVR and an equivalent ‘mock’ preparation were dissolved in 0.5 ml of 0.1 M-phosphate buffer pH 7, containing 0.15 M-NaCl (starting buffer), placed on a 2 ml column and recirculated for 45 to 60 min. The column was washed with 20 ml of starting buffer, and all the flow through fraction was collected. Elution was done with 0.5 M-glycine buffer pH 8.6.
Table 1. Purification of protoplast IVR by polyclonal antibody affinity chromatography*

<table>
<thead>
<tr>
<th>Sample</th>
<th>Elution buffer</th>
<th>Fraction no.</th>
<th>IVR</th>
<th>Control medium†</th>
<th>Inhibition of infectivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starting material§</td>
<td>0.1 M-Phosphate + 0.15 M-NaCl pH 7 0.5 M-Glycine pH 2.6</td>
<td>1</td>
<td>32.0 ± 4.1</td>
<td>34.1 ± 6.1</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>30.0 ± 5.0</td>
<td>32.0 ± 3.1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>13.8 ± 3.8</td>
<td>31.4 ± 2.3</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>15.1 ± 1.3</td>
<td>36.7 ± 2.5</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>11.1 ± 2.9</td>
<td>25.2 ± 2.3</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>29.0 ± 4.1</td>
<td>34.0 ± 5.7</td>
<td>15</td>
</tr>
<tr>
<td>Control protoplast‖</td>
<td>31.5 ± 2.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Column containing 9.2 mg IgG/g Sepharose.
† Average number of local lesions ± standard error per 10⁶ protoplasts on one half leaf of *Nicotiana glutinosa*, with the result normalized such that a standard TMV solution (0.2 μg/ml) yielded approximately 60 to 70 lesions per half leaf.
‡ Control preparation from medium in which uninoculated protoplasts were suspended.
§ Equivalent to 3 units.
‖ Control protoplasts without addition of IVR or control preparation.

2.6. Five 1 ml fractions were collected. All fractions were dialysed three times, against 2 l of double-distilled water, and freeze-dried. The resulting powder was dissolved in 50 to 100 μl of double-distilled water, and the biological activity of each fraction was tested in comparison with the respective control fractions (Gera & Loebenstein, 1983).

Three biologically active fractions were obtained (fractions 3, 4 and 5) (Table 1). The purity and integrity of the biologically active fractions were analysed by SDS-PAGE (Spiegel et al., 1989). A distinct band of 23K and three faint bands between 45K and 82K were visible in fraction 3 and were not present in the control preparation. (Fig. 1a). Fractions 4 and 5 also contained two close bands of 25K (not shown). Fraction 3 was freeze-dried and used for immunizing the mice.

A panel of MAbs directed against purified IVR was selected after fusion (Köhler & Milstein, 1975) of NS0 cells with spleen cells of a BALB/c mouse immunized with 5.5 μg of purified IVR (fraction 3). Identification of IVR-specific MAbs was achieved by ELISA (Clark & Adams, 1977) using Costar PVC plates coated with IVR or 'mock' preparations. Of 1300 hybridomas screened for specific antibody secretion nine gave specific ELISA reactions. These nine primary cultures were subcloned by limiting dilution (Moav et al., 1982) and three stable clones (MAbs-1, -2 and -3) were obtained. Subclone 1 gave the highest ELISA values with low non-specific reaction (0.65 compared with 0.06). Hybridoma cells were propagated as ascites tumours in pristane-primed BALB/c mice. Ascitic fluid was clarified by low speed centrifugation and tested against fractionated IVR-1. Again, subclone 1 gave the highest ELISA values with low-specific reactions (1.108 compared with 0.167 at a dilution of 1:100). To analyse the specificity of the MAbs further, crude protoplast IVR and 'mock' preparations were subjected to SDS-PAGE and analysed by immunoblotting (Towbin et al., 1979), using MAb-1. An immunoblot of crude IVR showed one 23K protein band that was not present in the control preparation (Fig. 1c). Similar results (not shown) were obtained when fractionated IVR-1 and -2 were analysed by immunoblotting. This, as well as data from SDS–PAGE, supports our previous suggestion that the fractionated IVR-2 is a dimer of IVR-1 (Loebenstein & Gera, 1981).

The possibility that reaction with the MAb eliminates the biological activity of crude protoplast IVR was tested. Polystyrene microplates were coated with 200 μl ascitic fluid or
Fig. 1. SDS-PAGE in 5 to 20% gradient gels and immunoblotting of IVR. (a, b) SDS-PAGE of immunoaffinity-purified protoplast IVR, using polyclonal serum (a) or MAb-1 (b). Ten units of IVR or equivalent 'mock' preparations are in each lane. Gels were stained with Coomassie Brilliant Blue. Samples were: lane 1, Mr standards phosphorylase B (92.5K), bovine serum albumin (66.2K), ovalbumin (45K), carbonic anhydrase (31K), soybean trypsin inhibitor (21.5K) and lysozyme (14.4K); lane 2, crude control preparation; lanes 3, crude protoplast IVR; lanes 4, effluent; lanes 5 and 6, purified IVR fraction and control, respectively. (c) Immunoblot of crude protoplast and tissue IVR. Lane 1, Mr standards subjected to SDS-PAGE and transferred to Immobilon transfer membrane (0-45 µm; Millipore) and stained with Coomassie Brilliant Blue. Lane 2, crude control from tissue; lane 3, crude tissue IVR, when 15 units of each preparation were used. Lanes 4 and 5 as lanes 2 and 3 in (b). (d) Immunoblot of immunoaffinity-purified IVR (10 units) using MAb-1 (lane 1) as for lane 5 in (b).

normal mouse serum diluted 1:20 in coating buffer. The plates were incubated for 4 h at 37 °C and washed with phosphate-buffered saline containing 0.05% Tween 20. Fifty µl of crude protoplast IVR (3 units) was then added to triplicate wells. After 3 h incubation, the fluid from the wells was collected and its biological activity was tested on TMV-infected tobacco protoplasts. Crude protoplast IVR placed on wells coated with normal mouse serum was used as a control. MAbs produced by clones 1, 2 and 3 decreased the biological activity of IVR by 77 ± 11% when compared with normal serum. When a further cycle of binding was performed, all inhibitory activity in the crude preparations was removed by binding to the MAbs.

MAb-1 was selected for immunoaffinity purification of crude protoplast IVR, using immunoglobulins from the ascitic fluid. IgGs were precipitated with 40% ammonium sulphate and coupled to CNBr-activated Sepharose 4B (10 mg/ml gel). A column of 2 ml volume was used. Fifty units of lyophilized crude protoplast IVR dissolved in 0.5 ml of starting buffer were applied to the affinity column and recirculated for 30 min. Proteins bound to the immobilized antibodies were eluted with 0.1 M-acetic acid pH 2.5 containing 0.5 M-NaCl, at a flow rate of two drops/min. Four fractions of 1 ml each were collected and neutralized with 30 µl of 2 M-Tris-HCl, dialysed for 48 h against double-distilled water, and treated as described above. Biological activity (> 56% inhibition) was observed in the fractions eluted with 0.1 M-acetic acid, whereas no biological activity could be detected in the flow through fraction. The recovery was almost quantitative (>90% based on biological activity and PAGE).

All four fractions of the immunoaffinity-purified IVR contained a single 23K protein, not present in the control preparation. This protein migrated to the same position as the specific band observed in PAGE of crude protoplast IVR (Fig. 1b) and reacted specifically in immunoblots with MAb-1 (Fig. 1d). Tissue IVR also reacted specifically with MAb in immunoblots and the reactive component comigrated with protoplast IVR (Fig. 1c). MAb-1 also reacted with tissue IVR isolated from the intercellular fluid of hypersensitive tobacco plants and these results strengthen our previous findings that tissue IVR and protoplast IVR are identical (Spiegel et al., 1989). The sensitivity of the MAbs enabled the detection of 0.1 to 1.0 ng
Short communication

IVR as measured by ELISA and these MAbs can therefore be used for both improved purification of IVR and its detection.

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REFERENCES


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