Primary Epitopes of Chicken Egg Yolk Antibodies to Peptidophosphogalactomannan†

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Egg yolks from hens immunized with peptidophosphogalactomannan (pPGalMan), which contains 10 phosphocholine diester residues and is secreted by Penicillium flettatum, contain antibodies against 5-β-D-galactofuranosyl epitopes. These epitopes were the only significant determinants in pPGalMan. Approximately 60-fold less pPGalMan (1.6 μM galactofuran chains) was required for 50% inhibition than galactofuranosyl-oligosaccharides or pPGalMan containing two galactofuranosyl residues per chain.

Filamentous fungi produce soluble extracellular polysaccharides and glycopeptides (1, 9, 10, 14, 19, 21, 23). Many of these polymers have active antigenic determinants (3, 14, 17, 20, 27). Penicillium flettatum (formerly Penicillium chartarutum) peptidophosphogalactomannans (pPGalMan; Mc, 25,000 to 70,000) (9, 19, 21, 23, 25, 32) contain a mannan with about 80 α-1,2- and α-1,6-mannopyranosyl residues and 12 small manno-oligosaccharidyl units, each attached to a 3-kDa peptide (Fig. 1). Eight to ten 5-β-D-galactofuranosyl-containing chains with 2 to 20 residues branch from the mannan. pPGalMan and pPGalMan (26, 31) contain approximately 10 and 2 phosphocholine diester residues, respectively, and a variable number of galactofuranosyl-6-O-phosphodiester residues (5).

Sera from rabbits immunized with whole-cell preparations from P. flettatum reacted with galactofuranosyl-containing heteropolysaccharide (20). Sera from guinea pigs injected with purified pPGalMan conjugated to bovine gamma globulin reacted weakly to manno-oligosaccharides of pPGalMan (11) and were unreactive to galactofuranosyl residues. Soluble pPGalMan did not elicit antibody in any of several species. This preparation, pPGalMan, was later shown to contain an average of two galactofuranosyl residues per galactan chain (unpublished data).

Antibodies that react specifically with furanosyl residues of parasites are of increasing clinical importance (4, 6–18). The purpose of this investigation was to determine if stable antibody could be elicited from purified glycopeptides, such as pPGalMan in phosphate-buffered saline (PBS) without adjuvant, and to determine the polymers’ epitope(s). Laying hens challenged with immunogenic substances during the laying season produce eggs that contain immunoglobulin Y (IgY), which is similar but not identical to IgG, in their yolks. Antibody is selectively deposited in egg yolk and is obtained by noninvasive means (2, 18).

Preliminary experiments. No immunological response was obtained in laying hens injected subcutaneously and in the footpad at weeks 1 and 3 with solutions of pPGalMan (200 μg/ml in PBS) and with whole P. flettatum cells at weeks 6 and 9. A response to subcutaneous injections of rabbit IgG in PBS was obtained in these hens. In contrast, other chickens responded to a course of two subcutaneous and two intravenous injections of either pPGalMan or pPGalMan in PBS. The immune responses to pPGalMan and pPGalMan were similar. Yolks from eggs stored at 4°C for a year retained antibody with little loss of activity. In these experiments, anti-pPGalMan activity was tested routinely by an enzyme-linked immunosorbent assay (ELISA) procedure (24, 27) in microtiter plates (Dynatech Laboratories, Inc.) coated with 0.4 μg (0.057 nmol) of either pPGalMan or pPGalMan (26) in 0.14 M NaCl-0.02% NaN, after incubation for 24 h at 4°C, the wells were washed with PBS containing 0.05% Tween 20. Unoccupied wells were blocked with 1 mg of bovine serum albumin in 0.1 ml of a solution of PBS, 0.01% NaN, and 0.05% Tween 20. Incubation at 24°C for 45 min followed. Plates were washed with PBS-NaN – Tween 20. Primary antibodies, prediluted with PBS, were added to all wells except those in the row that served as the secondary-antibody control. Plates were incubated for 60 min at 24°C. After the wells were washed, the quantity of chicken anti-pPGalMan antibody adsorbed to pPGalMan in each well was determined with rabbit anti-chicken IgG (whole molecule) alkaline phosphatase conjugate with p-nitrophenylphosphate as the substrate in 10% diethanolamine buffer (pH 9.8–0.2% NaN, p-Nitrophenol released in each well was quantified with a Bio-Rad ELISA model 2550 enzyme immunoassay reader set at 405 nm.

Purification of chicken egg yolk anti-P. flettatum antibody. Antibodies were fractionated by polyethylene glycol precipitation, hydrophobic-interaction chromatography, and gel permeation chromatography (12). Anti-pPGalMan activity from permeation chromatography resulted in a 31-fold increase in ELISA units per microgram of protein. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (13) showed anti-pPGalMan activity at 28 and 62 kDa.

Immunochemical studies. The reaction between pPGalMan or pPGalMan and anti-P. flettatum pPGalMan antibodies was quantified with 5 μg of protein/well. pPGalMan or pPGalMan (0 to 1 μg/well) was used in an indirect ELISA system. Both pPGalMan species bound to Immulon wells in a
hyperbolic concentration-dependent manner. Half saturation of the wells occurred with 26 nmol of either pPGalMan species (data not shown). Approximately 57 nmol (0.4 μg/well) of pPGalManii or pPGalManiii was used to coat the wells.

Competitive inhibition experiments with a range of concentrations of soluble phosphagalactomannan (PGalManii) or pPGalManii as the inhibitor of antibody interaction with bound pPGalManii or pPGalManiii, respectively, showed 50% inhibition at 0.14 and 0.16 μM (1.4 and 1.6 μM galactofuran chains), respectively (Table 1). This suggests that phosphocholine phosphodiester is a major epitope because pPGalManii, which contains at least fivefold more phosphocholine phosphodiester than pPGalManiii (26, 31), is not a better inhibitor than pPGalManiii. The epitope(s) on pPGalManii was determined with fragments derived by chemical or enzymatic degradation of pPGalManii. A range of concentrations of each fragment was tested as a hapten inhibitor of binding of anti-pPGalManiii antibodies to pPGalManii in a competitive ELISA inhibition system. The concentration of inhibitor or galactofuran chains required to inhibit 50% of antibody binding to Immulon-bound pPGalManii (Table 1) was determined from the plots of the percentages of inhibition versus log micromolar values of inhibition or chain.

TABLE 1. Inhibition of antibody binding to pPGalManii by modified pPGalManii and by oligosaccharide fragments

| Inhibitorb | 50% Inhibitory conc (μM) | Residues/ chain (n) |
|------------|--------------------------|---------------------|
| pPGalManii | 0.16 1.6 20              |                     |
| pPGalManii in PBS | 0.13 1.3 20              |                     |
| PGalMan    | 0.14 1.4 12              |                     |
| pPMan      | NI 11                  |                     |
| Peptide    | NI 11                  |                     |
| pP(Gal)_Man| 9.8 98                 | 2                   |
| Galactofuran-oligosaccharides |          |                     |
| Tetrasaccharide(s) | 55 55 | 4                   |
| Trisaccharide(s)  | 100 100 | 3                   |
| Disaccharide    | 180 180 | 2                   |
| 1-O-β-CH2-O-Man | 3,600 N/A |                     |
| Anionic saccharide | 125 125 | 2                   |

a NI, no inhibition; N/A, not applicable.
b Molecular masses are as follows: pPGalManii, 65 kDa; PGalMan, 62 kDa; pPMan, 18.6 kDa; and pP(Gal)_Man, 21.9 kDa.

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