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Primary Epitopes of Chicken Egg Yolk Antibodies to Peptidophosphogalactomannan†

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Egg yolks from hens immunized with peptidophosphogalactomannan (pPGalMan^{ii}), which contains 10 phosphocholine diester residues and is secreted by *Penicillium fellutanum*, contain antibodies against 5-*O*-β-D-galactofuranosyl epitopes. These epitopes were the only significant determinants in pPGalMan^{ii}. Approximately 60-fold less pPGalMan^{ii} (1.6 μM galactofuran chains) was required for 50% inhibition than galactofuranooligosaccharides 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 fellutanum* (formerly *Penicillium charlesii*) peptidophosphogalactomannans (pPGalMan; M_w, 25,000 to 70,000) (9, 19, 21, 23, 25, 26, 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-*O*-β-D-galactofuranosyl-containing chains with 2 to 20 residues branch from the mannan. pPGalMan^{ii} and pPGalMan^{iii} (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. fellutanum* 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^{ii} (11) and were unreactive to galactofuranosyl residues. Soluble pPGalMan did not elicit antibody in any of several species. This preparation, pP(Gal_2)Man, was later shown to contain an average of two galactofuranosyl residues per galactan chain (unpublished data).

Antisera from rabbits immunized with extracellular polysaccharides of *Penicillium* sp. cell walls react with synthetic β-D-galactofuranosyl-containing oligosaccharides (17). The 5-*O*-β-D-galactofuranosaccharides resulted in the most inhibition.

Antibodies that react specifically with furanosyl residues of parasites are of increasing clinical importance (4, 6–8, 14–16, 22, 28–30).

The purpose of this investigation was to determine if stable antibody could be elicited from purified glycopeptides, such as pPGalMan^{ii} 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^{ii} (200 μg/ml in PBS) and with whole *P. fellutanum* 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^{ii} or pPGalMan^{iii} in PBS. The immune responses to pPGalMan^{ii} and pPGalMan^{iii} 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^{ii} or pPGalMan^{iii} (26) in 0.14 M NaCl–0.02% NaN_3. 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_3, and 0.05% Tween 20. Incubation at 24°C for 45 min followed. Plates were washed with PBS-NaN_3-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^{ii} 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_3. *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. fellutanum* antibody. Antibodies were fractionated by polyethylene glycol precipitation, hydrophobic-interaction chromatography, and gel permeation chromatography (12). Anti-pPGalMan^{ii} 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^{ii} activity at 28 and 62 kDa.

Immunochemical studies. The reaction between pPGalMan^{ii} or pPGalMan^{iii} and anti-*P. fellutanum* pPGalMan antibodies was quantified with 5 μg of protein/well. pPGalMan^{ii} or pPGalMan^{iii} (0 to 1 μg/well) was used in an indirect ELISA system. Both pPGalMan species bound to Immulon wells in a

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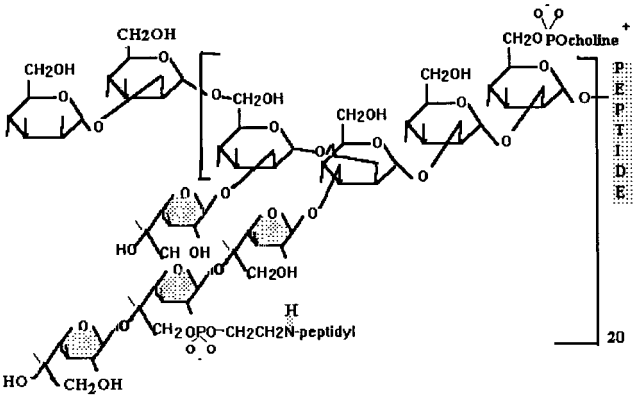

FIG. 1. Diagram of pPGalMan. The mannopyranosyl residues in each tetrasaccharide are attached by α-1,2 linkages and the tetrasaccharides are attached by α-1,6 linkages. The mannan is attached to the peptide by an *O*-glycosidic linkage to a seryl residue. Manno-oligosaccharides are attached to seryl and threonyl residues. An average of one galactan chain branches from each manno-octasaccharide and one phosphocholine phosphodiester is attached to C-6 of a mannopyranosyl residue.

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 pPGalMan^{ii} or pPGalMan^{iii} was used to coat the wells.

Competitive inhibition experiments with a range of concentrations of soluble phosphogalactomannan (PGalMan^{ii}) or pPGalMan^{ii} as the inhibitor of antibody interaction with bound pPGalMan^{ii} or pPGalMan^{iii}, 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 not a major epitope because pPGalMan^{ii}, which contains at least fivefold more phosphocholine phosphodiester than pPGalMan^{iii} (26, 31), is not a better inhibitor than pPGalMan^{iii}. The epitope(s) on pPGalMan^{ii} was determined with fragments derived by chemical or enzymatic degradation of pPGalMan^{ii}. A range of concentrations of each fragment was tested as a hapten inhibitor of binding of anti-pPGalMan^{ii} antibodies to pPGalMan^{ii} in a competitive ELISA inhibition system. The concentration of inhibitor or galactofuran chains required to inhibit 50% of antibody binding to Immulon-bound pPGalMan^{ii} (Table 1) was determined from plots of the percentages of inhibition versus log micromolar values of inhibition or chain.

TABLE 1. Inhibition of antibody binding to pPGalMan^{ii} by modified pPGalMan^{ii} and by oligosaccharide fragments[a]

Inhibitor[b]	50% Inhibitory concn (μM)		Residues/ chain (*n*)
	Saccharide	Galactofuran chains	
pPGalMan^{ii}	0.16	1.6	20
pPGalMan^{ii} in PBS	0.13	1.3	20
PGalMan	0.14	1.4	12
pPMan	NI	NI	N/A
Peptide	NI	NI	N/A
pP(Gal$_2$)Man	9.8	98	2
Galactofurano-oligosaccharides			
Tetrasaccharide(s)	55	55	4
Trisaccharide(s)	100	100	3
Disaccharide	180	180	2
1-*O*-β-CH$_3$-D-Man	3,600	N/A	N/A
Anionic saccharide	125	125	2

[a] NI, no inhibition; N/A, not applicable.
[b] Molecular masses are as follows: pPGalMan^{ii}, 65 kDa; PGalMan, 62 kDa; pPMan, 18.6 kDa; and pP(Gal)$_2$Man^{ii}, 21.9 kDa.

Peptide or peptidophosphomannan (pPMan), obtained by treatment of pPGalMan^{ii} with dilute acid, did not inhibit the immune response. In contrast, 9.8 μM pP(Gal$_2$)Man^{ii} (98 μM galactofuran chain) resulted in 50% inhibition of pPGalMan^{ii} binding to anti-pPGalMan^{ii}. Ten of 20 galactofuranosyl residues in pP(Gal$_2$)Man^{ii} are phosphodiesters (5). Considering that each chain contains two galactofuranosyl residues, the neutral galactofuranotrisaccharide binds with about the same avidity as the average of each chain in pP(Gal$_2$)Man^{ii}. This is further evidence that mannopyranosyl-6-*O*-phosphocholine phosphodiester in pPMan^{ii} is not a significant epitope. Furthermore, pPMan, which also contains phosphodiester residues, was not inhibitory.

Anionic galactofurano-oligosaccharide, obtained from an anion-exchange resin following dilute-acid treatment of pPGalMan^{ii}, was comparable to neutral galactofuranotrisaccharide as an inhibitor. Considered collectively, the data suggest that 5-*O*-β-D-galactofuranosyl residues are the primary epitopes in egg yolks from chickens challenged with pPGalMan^{ii}. The peptide region has no influence on antibody binding. The concentration of galactofurano-oligosaccharide or galactan chains in pP(Gal$_2$)Man^{ii} required for 50% inhibition of anti-pPGalMan^{ii} binding is more than 60-fold greater than that in pPGalMan^{ii} or PGalMan^{ii}. This suggests that galactofuran chains with a large number of residues have greater avidity for anti-pPGalMan^{ii}.

Although the chicken egg yolk anti-pPGalMan^{ii} antibody-antigen interaction in this study was not as sensitive as that from the rat on fungal galactomannan (27), the use of chickens may have utility in some situations in which antibody can be stored in the egg for long periods. The noninvasive means of obtaining antibody, the ease of isolation and purification of antibody, and the fact that adjuvant is not required to elicit significant antibody activity all may be of value in some situations.

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REFERENCES

1. **Bareto-Bergter, E., P. A. J. Gorin, and L. R. Travassos.** 1981. Cell constituents of mycelia and conidia of *Aspergillus fumigatus*. Carbohydr. Res. **95:** 205–218.
2. **Bar-Joseph, M., and M. Malkinson.** 1980. Hen egg yolk as a source of antiviral antibodies in the enzyme-linked immunosorbent assay (ELISA): a comparison of two plant viruses. J. Virol. Methods **1:**179–183.
3. **Bennett, J. E., A. K. Bhattacharjee, and C. P. J. Glaudemans.** 1985. Galactofuranosyl groups are immunodeterminant in *Aspergillus fumigatus* galactomannans. Mol. Immunol. **22:**251–254.
4. **Bhattacharjee, A. K., J. E. Bennett, and C. P. J. Glaudemans.** 1984. Capsular polysaccharides of *Cryptococcus neoformans*. Rev. Infect. Dis. **6:**619–623.
5. **Bonetti, S. J., L. T. Gelbaum, and J. E. Gander.** 1990. Peptide-containing phosphodiesters in *Penicillium* exocellular glycopeptide. FASEB J. **4:**A2305. (Abstract 3536.)
6. **De Arruda, V. M., W. Coli, and B. Zingales.** 1989. Terminal β-D-galactofuranosyl epitopes recognized by the antibodies that inhibit *Trypanosoma cruzi* internalization into mammalian cells. Eur. J. Biochem. **182:**413–421.
7. **Ferguson, M. A. J., and F. A. Williams.** 1988. Cell-surface anchoring of pro-

teins via glycosyl-phosphatidylinositol structures. Annu. Rev. Biochem. **57:** 285–320.
8. **Fincher, G. B., B. A. Stone, and A. E. Clarke.** 1983. Arabinogalactan-protein: structure, biosynthesis and function. Annu. Rev. Plant Physiol. **34:**47–70.
9. **Gander, J. E., N. H. Jentoft, L. R. Drewes, and P. D. Rick.** 1974. The 5-O-β-D-galactofuranosyl-containing exocellular glycopeptide of *Penicillium charlesii*. Characterization of peptidophosphogalactomannan. J. Biol. Chem. **249:** 2063–2072.
10. **Gander, J. E.** 1974. Fungal cell wall glycoproteins and peptidopolysaccharides. Annu. Rev. Microbiol. **28:**103–119.
11. **Gander, J. E., and J. A. Rudbach.** 1973. Immunological investigations of *Penicillium*. II. Primary binding of glycopeptide derivatives to specific antibodies. Immunochemistry **10:**81–92.
12. **Hassl, A., and H. Aspock.** 1988. Purification of egg yolk immunoglobulins. A two step procedure using hydrophobic interaction chromatography and gel filtration. J. Immunol. Methods **110:**225–228.
13. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
14. **Latgé, J.-P., H. Kobayashi, J.-P. Debeaupuis, M. Diaquin, J. Sarfati, J.-M. Wieruszeski, E. Parra, J.-P. Bouchara, and B. Fournet.** 1994. Chemical and immunological characterization of the extracellular galactomannan of *Aspergillus fumigatus*. Infect. Immun. **62:**5424–5433.
15. **McNeil, M. R., and P. J. Brennan.** 1991. Structure, function and biogenesis of the cell envelope of mycobacteria in relation to bacterial physiology, pathogenesis and drug resistance; some thoughts and possibilities arising from recent structural information. Res. Microbiol. **142:**451–463.
16. **Meyer, R. D., L. S. Young, D. Armstrong, and B. Yu.** 1973. Aspergillosis complicating neoplastic disease. Am. J. Med. **54:**6–15.
17. **Notermans, S., G. H. Veeneman, C. W. E. M. van Zuylen, P. Hoogerhout, and J. H. van Boom.** 1988. (1→5)-linked β-D-galactofuranosides are immunodominant in extracellular polysaccharides of *Penicillium* and *Aspergillus* species. Mol. Immunol. **25:**975–979.
18. **Polson, A., M. B. von Wechmar, and M. H. V. van Regenmortel.** 1980. Isolation of viral IgY antibodies from yolks of immunized hens. Immunol. Commun. **9:**475–493.
19. **Preston, J. F., E. Lapis, and J. E. Gander.** 1969. Isolation and partial characterization of the exocellular polysaccharides of *Penicillium charlesii*. II. The occurrence of phosphate groups in high molecular weight polysaccharides. Arch. Biochem. Biophys. **134:**316–323.
20. **Preston, J. F., E. Lapis, and J. E. Gander.** 1970. Immunological investigations of *Penicillium*. I. Serological reactivities of exocellular polysaccharides produced by six *Penicillium* species. Can. J. Microbiol. **16:**687–694.
21. **Preston, J. F., E. Lapis, S. Westerhouse, and J. E. Gander.** 1969. Isolation and partial characterization of the exocellular polysaccharides of *Penicillium charlesii*. III. Heterogeneity in size and composition of high molecular weight polysaccharides. Arch. Biochem. Biophys. **134:**324–334.
22. **Reiss, E., and P. F. Lehmann.** 1979. Galactomannan antigenemia in invasive aspergillosis. Infect. Immun. **25:**357–365.
23. **Rick, P. D., L. R. Drewes, and J. E. Gander.** 1974. The 5-O-β-D-galactofuranosyl-containing glycopeptide of *Penicillium charlesii*. Occurrence of ethanolamine and partial characterization of peptide portion and the carbohydrate-peptide linkage. J. Biol. Chem. **249:**2073–2078.
24. **Ricke, S. C., D. M. Schaefer, M. E. Cook, and K. H. Kang.** 1988. Differentiation of ruminal bacterial species by enzyme-linked immunosorbent assay using egg yolk antibodies from immunized chicken hens. Appl. Environ. Microbiol. **54:**596–599.
25. **Rietschel-Berst, M., N. H. Jentoft, P. D. Rick, C. Pletcher, F. Fang, and J. E. Gander.** 1977. Extracellular exo-β-D-galactofuranosidase from *Penicillium charlesii*. Isolation, purification, and properties. J. Biol. Chem. **252:**3219–3224.
26. **Salt, S. D., and J. E. Gander.** 1985. Variations in phosphoryl substituents in extracellular peptidophosphogalactomannans from *Penicillium charlesii* G. Smith. Exp. Mycol. **9:**9–19.
27. **Stynen, D., A. Goris, J. Sarfati, and J. P. Latgé.** 1995. A new sensitive sandwich enzyme-linked immunosorbent assay to detect galactofuran in patients with invasive aspergillosis. J. Clin. Microbiol. **33:**497–500.
28. **Suzuki, S., and N. Takada.** 1975. Serological cross reactivity of the D-galacto-D-mannans isolated from several pathogenic fungi against anti-*Hormodendrum pedrosoi* serum. Carbohydr. Res. **40:**193–197.
29. **Swanink, C. M. A., J. F. G. M. Meis, A. J. M. M. Rijs, J. P. Donnelly, and P. E. Verweij.** 1997. Specificity of a sandwich enzyme-linked immunosorbent assay for detecting *Aspergillus* galactomannan. J. Clin. Microbiol. **35:**257–260.
30. **Turco, S. J., and A. Descoteaux.** 1992. The lipophosphoglycan of *Leishmania* parasites. Annu. Rev. Microbiol. **46:**65–94.
31. **Unkefer, C. J., C. L. Jackson, and J. E. Gander.** 1982. The 5-O-β-D-galactofuranosyl-containing glycopeptide from *Penicillium charlesii*. Identification of phosphocholine attached to C-6 of mannopyranosyl residues of the mannan region. J. Biol. Chem. **257:**2491–2497.
32. **Unkefer, C. J., and J. E. Gander.** 1990. The 5-O-β-D-galactofuranosyl-containing glycopeptide from *Penicillium charlesii*. Characterization of the mannan by ^{13}C NMR spectroscopy. J. Biol. Chem. **265:**685–689.