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Glycolipid binding epitopes involved in adherence of the periodontitis-associated bacterium *Porphyromonas gingivalis*

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Abstract The ability of the periodontal pathogen *Porphyromonas gingivalis* to use different glycolipid structures as receptors has previously been demonstrated. The bacterium adhered to acid and nonacid glycolipids originating from human organs and to nonacid glycolipids of porcine origin. The aim of the present study was to analyze these binding epitopes by structural characterization. Glycolipid fractions with positive bacterial binding from *e.g.* human and porcine origin, were purified by the high performance liquid chromatography technique and thereafter used in bacterial overlay assays with ^{35}S-labeled *P. gingivalis*. Purified fractions with positive binding were structurally characterized by proton nuclear magnetic resonance spectroscopy. Complementing thin-layer chromatograms and bacterial overlay assays with pure reference glycolipid fractions and competition experiments with lactose were performed to define potential receptors. The *P. gingivalis* binding epitopes, including cerebrosides with nonhydroxy fatty acids, lactosylceramide with hydroxy fatty acids, sulfatides, lacto-, neolacto- and gangliotetraosylceramides, are in several instances similar to those found for other bacteria, *e.g. H. pylori*, *H. influenzae* and *N. meningitidis*. In addition *P. gingivalis* also bound to the Galα4Gal epitope of the globo series of glycolipids. In the future these results may be valuable for development of new treatment strategies, such as anti-adhesion therapies and vaccines specifically directed against *P. gingivalis* infection.

Keywords Adhesion · Glycolipid · NMR · *Porphyromonas gingivalis* · Structural characterization

Abbreviations

BSA	bovine serum albumin
hFA	hydroxy fatty acid
HPLC	high performance liquid chromatography
nFA	non-hydroxy fatty acid
NMR	nuclear magnetic resonance spectroscopy
P	phytosphingosine
PBS	phosphate buffered saline
S	sphingosine

Introduction

The anaerobic periodontal pathogen *Porphyromonas gingivalis* has the ability to adhere to and invade oral epithelial cells and human pocket epithelium *in vitro* [46–47]. This is a crucial virulence trait that provides the bacterium with a temporary shelter against antibodies and phagocytic cells of the host. The adhesin(s) of *P. gingivalis* has been in main focus in the majority of the previous studies concerning the adhesion process. It has, among other things, been shown that the bacterium uses its fimbriae, when adhering to cell

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surfaces. The *fimA* gene encoding the structural protein fimbrillin has been isolated [13, 33] and peptides with the bacterial fimbrillin amino acid sequence have been synthesized and used to abolish bacterial adhesion to saliva-coated hydroxyl apatite [34]. It is also known that *P. gingivalis* strains, which express only a minor arsenal of fimbriae or no fimbriae at all, show a decreased binding capacity to epithelial cells [34]. However, less is known about the structure of the cell surface receptor(s) that the fimbriae adhere(s) to.

Glycoproteins exist in different forms in the membranes of eukaryotic cells. A characteristic feature of glycoproteins is the dominating protein moiety of the molecule, compared to proteoglycans, which consist of a dominating carbohydrate part. Carbohydrates existing on glycoproteins can often also be found on glycolipids on the cell surface. The carbohydrate epitopes on glycolipids and glycoproteins are in many cases identical, but the core chains do vary [42]. When compared to glycoproteins, the core chains on glycolipids are generally shorter and less branched. They are also quite distinct from the core sequences of glycoproteins [60]. The expression of carbohydrate chains can also vary between different animal species [4, 7, 50, 58], between different organs [5, 6, 57], and also between different cells [52].

It has been shown that a number of bacteria bind to glycoconjugates on cell surfaces [31, 39], *e.g. Streptococcus suis* [15], *Escherichia coli* [14, 27] and *Helicobacter pylori* [3, 9, 55]. Theories and studies based on various proteins including glycosylated proteins have been published, but none has yet been able to characterize any structure pertaining to the bacterial binding epitope(s) of *P. gingivalis*. *In vitro* studies using glycoprotein from oral epithelium suggest that glycoconjugates acts like bacterial receptors [2]. This study also showed that addition of acidic monosaccharides such as sialic acid or glucoronic acid to a bacterial suspension abolishes binding of *P. gingivalis* to oral epithelial cells [2]. This fact suggests that carbohydrate moieties somehow are involved in the adhesion process. Previous studies have also shown that *P. gingivalis* can hydrolyze proteins and thereby expose previously hidden epitopes (*i.e.*, cryptitopes) [32]. This strategy might be adopted by the bacterium, when adhering to carbohydrate epitopes on glycolipids as well.

In a previous study, we were able to show that *P. gingivalis* has the ability to bind to total glycolipid fractions originating from human and porcine organs [20] by screening of a large array of glycolipid structures with the thin-layer chromatogram binding assay. Binding was obtained to both acid and nonacid glycolipids of human origin and to nonacid glycolipids of porcine origin. We thus found that sulfated glycolipids probably are favored by the bacterium in acid glycolipid fractions, whereas cerebrosides and glycolipids based on the globo series seemed to be preferred in the nonacid glycolipid fractions.

The aim of the present study was to further purify and structurally characterize putative receptors of *P. gingivalis*. Based on results from proton nuclear magnetic resonance (^{1}H NMR) analysis, bacterial overlay assays with pure reference glycolipids and competition experiments with lactose, potential bacterial receptors with different binding epitopes were identified.

Results

Bacterial overlay assays

Total acid glycolipid fractions from human kidney and total nonacid glycolipid fractions from porcine small intestine, salivary gland, kidney, liver, and spleen were further purified and fractionated by silicic acid column chromatography on a high performance liquid chromatography (HPLC) system. The purified fractions were subsequently separated by thin-layer chromatography (TLC) and used in a bacterial overlay assay with ^{35}S-labeled *P. gingivalis* FDC381. When visual inspection of the autoradiograms was performed to evaluate potential binding patterns, we observed that the bacterium favored adhesion to the fast moving lanes containing shorter glycolipids (Figs. 1, 2, 3 and 4).

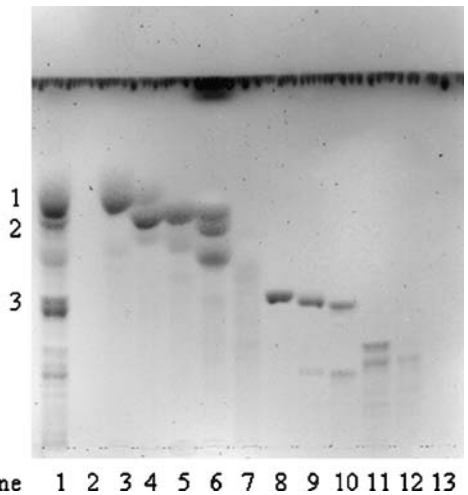

Fig. 1 Thin layer chromatogram of acid glycolipid fractions from human kidney with positive binding of ^{35}S-labeled *Porphyromonas gingivalis*. Detection of glycolipids with anisaldehyde reagent. Glycolipids (2–10 μg) were added to the lanes (*1–13*). The solvent system was chloroform/methanol/water with 0.2% $CaCl_2$ (60:40:9 by volume). The lanes contained total glycolipid fraction from human kidney (*lane 1*) and pooled fractions after purification by HPLC technique (*lanes 2–13*). The number of sugar residues in the glycolipid chains is indicated on the *left side*

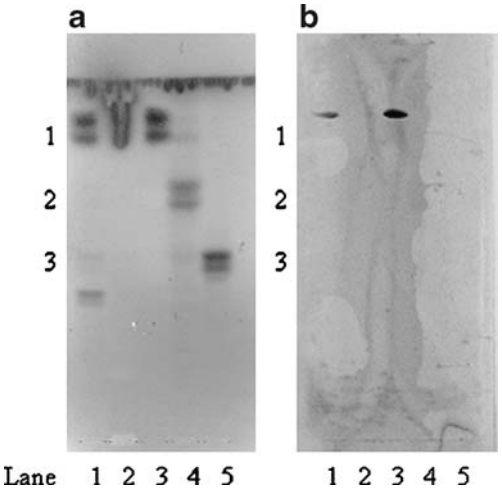

Fig. 2 Thin layer chromatogram of nonacid glycolipid fractions from porcine salivary gland with positive binding of ^{35}S-labeled *Porphyromonas gingivalis*. **a** Detection of glycolipids with anisaldehyde reagent. **b** Binding pattern of the bacteria visualized by autoradiography. The binding assay was performed as described in "Materials and methods." 2–10 μg of the glycolipids were added to the lanes (*1–5*). The solvent system was chloroform/methanol/water (60:35:8 by volume). The lanes contained total glycolipid fraction from porcine salivary gland (lane 1) and pooled fractions after purification by HPLC technique (*lanes 2–5*). The number of sugar residues in the glycolipid chains is indicated on the *left side*

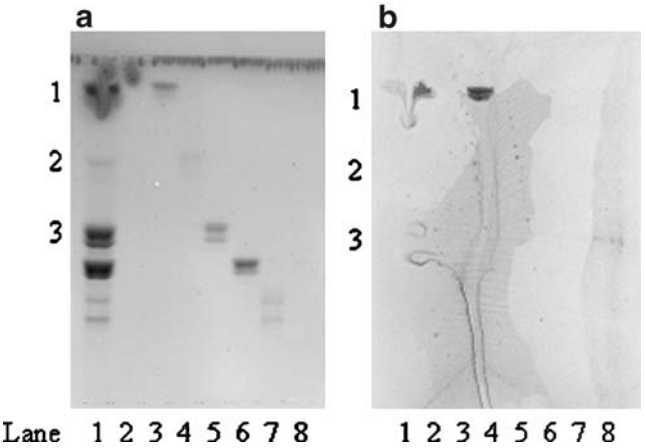

Fig. 4 Thin layer chromatogram of nonacid glycolipid fractions from porcine spleen with positive binding of ^{35}S-labeled *Porphyromonas gingivalis*. **a** Detection of glycolipids with anisaldehyde reagent. **b** Binding pattern of the bacteria visualized by autoradiography. The binding assay was performed as described in "Materials and methods." 2–10 μg of the glycolipids were added to the lanes (*1–8*). The solvent system was chloroform/methanol/water (60:35:8 by volume). The lanes contained total glycolipid fraction from porcine spleen (*lane 1*) and pooled fractions after purification by HPLC technique (*lanes 2–8*). The number of sugar residues in the glycolipid chains is indicated on the *left side*

Figure 1 thus shows acid glycolipids from human kidney. Binding was detected in the one- and two-sugar region (not shown). The glycolipids were subsequently identified (see "Glycolipid characterization by NMR") as sulfatides, *i.e.* SO_3-3Galβ1Cer and SO_3-3Galβ4Glcβ1Cer. Figures 2, 3 and 4 show binding to nonacid glycolipids from porcine salivary gland, kidney and spleen all revealing binding in the 1-sugar region only. All three sources contained Glcβ1Cer with both hydroxy and nonhydroxy fatty acids except for porcine spleen having only nonhydroxy fatty acids, whereas salivary gland also contained Galβ1Cer with both types of fatty acid (see below). The porcine spleen fraction was binding positive, indicating that Glcβ1Cer with nonhydroxy fatty acids are a requirement for binding to occur.

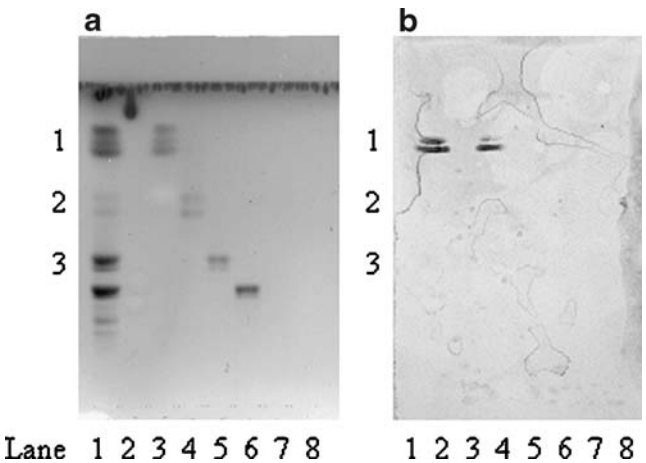

Fig. 3 Thin layer chromatogram of nonacid glycolipid fractions from porcine kidney with positive binding of ^{35}S-labeled *Porphyromonas gingivalis*. **a** Detection of glycolipids with anisaldehyde reagent. **b** Binding pattern of the bacteria visualized by autoradiography. The binding assay was performed as described in "Materials and methods." 2–10 μg of the glycolipids were added to the lanes (*1–8*). The solvent system was chloroform/methanol/water (60:35:8 by volume). The lanes contained total glycolipid fraction from porcine kidney (*lane 1*) and pooled fractions after purification by HPLC technique (*lanes 2–8*). The number of sugar residues in the glycolipid chains is indicated on the *left side*

Also Galβ1Cer with nonhydroxy fatty acids is binding positively as shown by complementing bacterial overlay assays with pure reference glycolipid fractions (Fig. 5, lane 1). To these results binding to lactosylceramide with hydroxy fatty acids from dog small intestine (lane 2), but not to lactosylceramide with nonhydroxy fatty acids from human granulocytes (lane 3), can be added. Furthermore, weak binding to lactotetraosylceramide (Galβ3GlcNAcβ3Galβ4Glcβ1Cer) from human meconium (lane 4) and isoglobotriaosylceramide (Galα3Galβ4Glcβ1Cer) from dog small intestine (lane 5) is also seen, whereas binding to sulfgangliotetraosylceramide (SO_3-3Galβ3GalNAcβ4Galβ4Glcβ1Cer) (lane 6) and various ganglioside structures (lanes 7 and 8) is not found. In addition, binding to galabiosylceramide (Galα4Galβ1Cer), globotriaosylceramide (Galα4Galβ4Glcβ1Cer) and globotetraosylceramide (GalNAcβ3Galα4Galβ4Glcβ1Cer) are found (not shown)

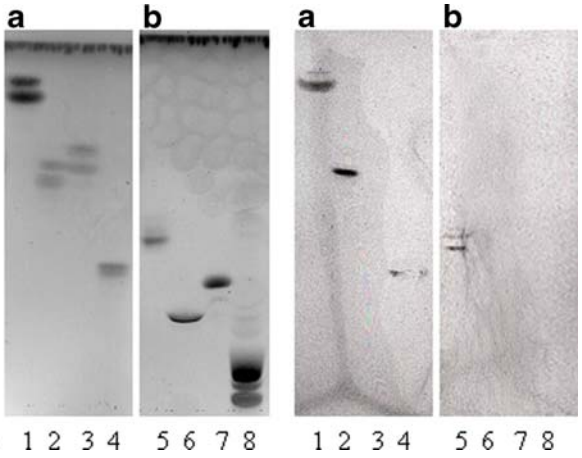

Fig. 5 Thin layer chromatograms of pure reference glycolipid fractions with positive binding of ^{35}S-labeled *Porphyromonas gingivalis*. **a** Detection of glycolipids with anisaldehyde reagent. **b** Binding pattern of the bacteria visualized by autoradiography. The binding assay was performed as described in "Materials and methods." 4 μg of the glycolipids were added to *lane 1–7* and 40 μg to *lane 8*. The solvent system was chloroform/methanol/water (60:35:8, by volume). The lanes galactocerebrosides from bovine brain (*lane 1*), lactosylceramide from dog small intestine (*lane 2*), lactosylceramide from human granulocytes (*lane 3*), lactotetraosylceramide from human meconium (*lane 4*), isoglobotriaosylceramide from dog small intestine (*lane 5*), sulfated gangliotetraosylceramide from mouse faeces (*lane 6*), ganglioside (GM3) from human brain (*lane 7*), and ganglioside mixture (GM3, GM1, GD1a and GD1b) from calf brain (*lane 8*)

as summarized in Table 1. Moreover, in our previous study, binding to gangliotriaosylceramide (GalNAcβ4 Galβ4Glcβ1Cer) and gangliotetraosylceramide (Galβ3Gal NAcβ4Galβ4Glcβ1Cer) was obtained [20].

De-*N*-acylation of neolactotetraosylceramide

In Fig. 6 it is shown that binding to neolactotetraosylceramide (Galβ4GlcNAcβ3Galβ4Glcβ1Cer) occurs (lane 3). In order to verify whether the terminal segment of the neolactotetraosylceramide is part of the binding epitope or not de-*N*-acylation of the GlcNAcβ3 residue to $GlcNH_2$β3 was performed [3]. The result of the bacterial overlay assay (lane 4) reveals that no binding could be detected to the de-*N*-acylated form of the glycolipid, indicating involvement of the terminal disaccharide in binding to *P. gingivalis*.

Glycolipid characterization by NMR

The lanes that contained glycolipid fractions with positive bacterial binding where structurally characterized by ^{1}H NMR. Data from the NMR spectral analyses of the various fractions used in the present study are summarized in Table 2 and are briefly described in the following. Selected spectra are shown in Fig. 7.

Analysis of the NMR spectra of acid glycolipids from human kidney showed that the one-sugar fraction (Fig. 1, lane 3) contains 3-sulfated galactosylceramide having a sphingosine base and either a hydroxy (hFA) or non-hydroxy (nFA) fatty acid as evidenced by the shifts of two Galβ1 anomeric proton resonances having approximately equal intensity at 4.182 and 4.154 ppm, respectively, values typical for sulfatide [25]. The next fractions (Fig. 1, lanes 4 and 5; Fig. 7b), contains two sugars whose anomeric resonances are found at 4.315 (SO_3-3Galβ4), 4.178 (Glcβ1, hFA) and 4.156 ppm (Glcβ1, nFA) thus corresponding to 3-sulfated lactosylceramide with a sphingosine base [41]. A somewhat later fraction (Fig. 1, lane 6) is found to exclusively contain SO_3-3Galβ4Glcβ1Cer with a sphingosine base and nFA.

An acid one-sugar fraction, also from a human kidney (data not shown), contains 3-sulfated galactosylceramide having both a sphingosine (S) or phytosphingosine (P) base combined with either hFA or nFA, resulting in four anomeric resonances from the galactosyl residue at 4.229 (P + hFA), 4.215 (P + nFA), 4.179 (S + hFA) and 4.152 ppm (S + nFA), where the two latter species dominate over the two former ones [25]. As for the latter kidney fraction also this one displays a fraction (data not shown) with 3-sulfated lactosylceramide having a sphingosine base and nFA as evidenced by anomeric resonances at 4.315 (SO_3-3Galβ4) and 4.157 ppm (Glcβ1) [41].

The dihexosylceramide fraction from porcine small intestine (Fig. 7c) reveals an α-signal at 4.810 ppm typical for Galα4 and two β-signals at 4.126 and 4.104 ppm originating from Galβ1 attached to a ceramide having hFA (minor) or nFA (major), respectively, thus identifying this structure as galabiosylceramide, Galα4Galβ1Cer [21]. The next fraction (Fig. 7d) reveals anomeric signals typical for globotriaosylceramide at 4.787 (Galα4), 4.261 (Galβ4) and 4.166 ppm (Glcβ1) with a sphingosine base and nFA [44], while the succeeding fraction (Fig. 7e) exhibits anomeric resonances originating from globotetraosylceramide with signals at 4.517 (GalNAcβ3), 4.803 (Galα4), 4.263 (Galβ4) and 4.167 ppm (Glcβ1) also with a sphingosine base and nFA [44].

A nonacid one-sugar fraction from porcine salivary gland (Fig. 2, lane 3) reveals a mixture Glcβ1Cer and Galβ1Cer as evidenced by anomeric β-signals at 4.081 ppm (nFA) for the former structure and at 4.069 (hFA) and 4.029 ppm (nFA) for the latter one [12, 59]. The presence of Glcβ1Cer is further supported by the Glc H2 resonance seen at 2.961 ppm.

The first nonacid fraction from porcine kidney (Fig. 3, lane 3) contains a single one-sugar structure identifiable as Glcβ1Cer from the anomeric β-signal at 4.079 ppm (hFA) and the Glc H2 resonance seen at 2.961 ppm [12, 59]. The following fraction (Fig. 3, lane 4) is a mixture of two two-sugar compounds easily identified as galabiosylceramide (Galα4 at 4.809 ppm and Galβ1 at 4.104 ppm [nFA]) and

Table 1 Binding of *Porphyromonas gingivalis* to glycolipids on thin-layer chromatograms

Trivial name[a]	Glycolipid structure	Binding[b]	Source	References
Cerebroside (S + nFA)	Galβ1Cer	++	Bovine brain	[19]
Cerebroside (S + hFA)	Galβ1Cer	−	Bovine brain	[19]
Cerebroside (S + nFA)	Glcβ1Cer	++	Porcine spleen	[22]
LacCer (S + nFA)	Galβ4Glcβ1Cer	−	Human granulocytes	[36]
LacCer (S/P + hFA)	Galβ4Glcβ1Cer	++	Dog small intestine	[17]
Sulfatide (S/P + nFA/hFA)	SO_3-3Galβ1Cer	+	Human kidney	[23]
SO_3-3LacCer (S/P + nFA)	SO_3-3Galβ4Glcβ1Cer	+	Human kidney	[23]
Galabioside (S/P + hFA)	Galα4Galβ1Cer	+	Porcine small intestine	[4]
Globotri (S + nFA)	Galα4Galβ4Glcβ1Cer	(+)	Porcine small intestine	[4]
Globotetra (S + nFA)	GalNAcβ3Galα4Galβ4Glcβ1Cer	(+)	Porcine small intestine	[4]
Isoglobotri	Galα3Galβ4Glcβ1Cer	+	Dog small intestine	[17]
GgO3 (S + nFA)	GalNAcβ4Galβ4Glcβ1Cer	+	Guinea pig erythrocytes	[48]
GgO4	Galβ3GalNAcβ4Galβ4Glcβ1Cer	+	Mouse small intestine	[18]
SO_3-3GgO4	SO_3-3Galβ3GalNAcβ4Galβ4Glcβ1Cer	−	Mouse small intestine	[35]
NeuAc-GM3 (S + nFA)	NeuAcα3Galβ4Glcβ1Cer	−	Human brain	[54]
Lactotetra (S/P + hFA)	Galβ3GlcNAcβ3Galβ4Glcβ1Cer	+	Human meconium	[55]
Neolactotetra	Galβ4GlcNAcβ3Galβ4Glcβ1Cer	(+)	Human granulocytes	[36]
Ganglioside mixture			Calf brain	[54]
GM3	NeuAcα3Galβ4Glcβ1Cer	−		
GM1	Galβ3GalNAcβ4(NeuAcα3)Galβ4Glcβ1Cer	−		
GD1a	NeuAcα3Galβ3GalNAcβ4(NeuAcα3)-Galβ4Glcβ1Cer	−		
GD1b	Galβ3GalNAcβ4(NeuAcα8NeuAcα3)-Galβ4Glcβ1Cer	−		

[a] The abbreviations after the trivial name of glycolipids refer to the major ceramide species in the fractions utilized for binding studies, as determined by negative ion FAB mass spectrometry of native glycolipid fractions, and/or EI mass spectrometry of permethylated or permethylated/$LiAlH_4$-reduced derivatives or by proton NMR spectroscopy. S and P stand for sphingosine and phytosphingosine, respectively, while nFA and hFA designate nonhydroxy and hydroxy fatty acids, respectively.

[b] Significant darkening observed by visual inspection of the autoradiogram. + + indicates very strong binding, + indicates strong binding, (+) means that no binding was detected occasionally, and − indicates that no binding could be detected.

lactosylceramide (Galβ4 at 4.200 ppm and Glcβ1 at 4.161 ppm [nFA]) [21]. The Glc H2 resonance seen at 3.039 ppm is typical for 4-substituted glucose. The single glycolipid in the third fraction (Fig. 3, lane 5) reveals anomeric signals at 4.786 (Galα4), 4.253 (Galβ4) and 4.161 ppm (Glcβ1, nFA), which are identical to those of porcine small intestine, thus identifying the structure as globotriaosylceramide (Galα4Galβ4Glcβ1Cer) [44].

Thereafter, two nonacid glycolipid fractions from porcine liver were analyzed. The first, a one-sugar fraction (data not shown), reveals a mixture Glcβ1Cer and Galβ1Cer as evidenced by anomeric β-signals at 4.080 ppm (nFA) for the former structure and at 4.029 ppm (nFA) for the latter one [12, 59]. The presence of Glcβ1Cer is further supported by the Glc H2 resonance seen at 2.971 ppm. The next fraction (data not shown) consists of globotriaosylceramide with anomeric signals at 4.786 (Galα4), 4.250 (Galβ4) and 4.158 (Glcβ1) with a sphingosine base and nFA [44], practically identical to the values found in porcine small intestine.

Finally, two nonacid glycolipids from porcine spleen were analyzed. The first fraction (Fig. 4, lane 3; Fig. 7a) contains a single one-sugar structure identifiable as Glcβ1Cer from

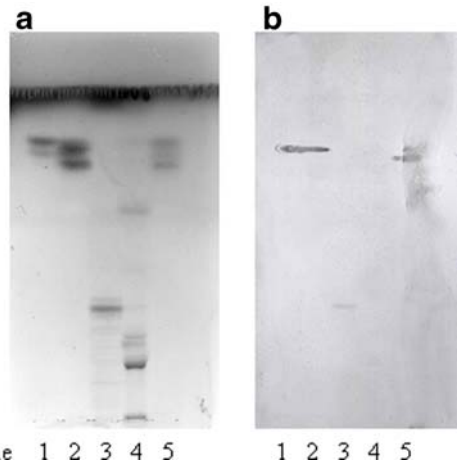

Fig. 6 Thin layer chromatogram after de-*N*-acylation. **a** Detection of glycolipids with anisaldehyde reagent. **b** Binding pattern of the bacteria visualized by autoradiography. The binding assay was performed as described in "Materials and methods." 4–8 μg of the glycolipids were added to *lane 1–5*. The solvent system was chloroform/methanol/water (60:35:8 by volume). The lanes contained Glcβ1Cer from porcine spleen (*lane 1*), Galβ1Cer and Glcβ1Cer from porcine salivary gland (*lane 2*), Galβ4GlcNAcβ3Galβ4Glcβ1Cer from human granulocytes (*lane 3*), de-*N*-acylated Galβ4GlcNAcβ3Galβ4Glcβ1Cer (*lane 4*), and Glcβ1Cer from porcine kidney (*lane 5*)

Table 2 Structural identification of glycolipids from different sources of human and porcine origin by ^{1}H NMR spectroscopy

Trivial name[a]	Structures	Ceramide structure[b]	References
Fractions from human kidney			
Sulfatide	SO_3-3Galβ1Cer	S + nFA and P + nFA	[23]
Sulfated lactosylceramide	SO_3-3Galβ4 Glcβ1Cer	S + nFA and P + nFA	[23]
Sulfated lactosylceramide	SO_3-3Galβ4 Glcβ1Cer	S + nFA	[23]
Sulfatide	SO_3-3Galβ1Cer	S/P + nFA/hFA	[23]
Sulfated lactosylceramide	SO_3-3Galβ4 Glcβ1Cer	S + nFA	[23]
Fractions from porcine small intestine			
Galabiosylceramide	Galα4Galβ1Cer	S + nFA (major) and S + hFA (minor)	[4]
Globotriaosylceramide	Galα4Galβ4 Glcβ1Cer	S + nFA	[4]
Globotetraosylceramide	GalNAc β3Galα4Galβ4 Glcβ1Cer	S + nFA	[4]
Fraction from porcine salivary gland			
Cerebroside	Galβ1Cer + Glcβ1Cer	S + nFA (Glc/Gal) and S + hFA (Gal)	[5]
Fractions from porcine kidney			
Cerebroside	Glcβ1Cer	S + nFA (major) and S + hFA (minor)	[5]
Lactosyl- and Galabiosylceramide	Galβ4 Glcβ1Cer + Galα4 Galβ1Cer	S + nFA	[5]
Globotriaosylceramide	Galα4Galβ4 Glcβ1Cer	S + nFA	[5]
Fractions from porcine liver			
Cerebroside	Galβ1Cer + Glcβ1Cer	S + nFA (Glc/Gal)	[5]
Globotriaosylceramide	Galα4Galβ4 Glcβ1Cer	S + nFA	[5]
Fractions from porcine spleen			
Cerebroside	Glcβ1Cer	S + nFA	[5]
Globotriaosylceramide	Galα4Galβ4 Glcβ1Cer	S + nFA	[5]

[a] The abbreviations after the trivial name of glycolipids refer to the major ceramide species in the fractions utilized for binding studies, as determined by negative ion FAB mass spectrometry of native glycolipid fractions, and/or EI mass spectrometry of permethylated or permethylated/$LiAlH_4$-reduced derivatives or by ^{1}H NMR spectroscopy.
[b] S and P stand for sphingosine and phytosphingosine, respectively, while nFA and hFA designate nonhydroxy and hydroxy fatty acids, respectively.

the anomeric β-signal at 4.080 ppm (nFA) and the Glc H2 resonance seen at 2.971 ppm [59], whereas the subsequent fraction (Fig. 4, lane 5) consists of globotriaosylceramide with anomeric signals at 4.787 (Galα4), 4.258 (Galβ4) and 4.164 ppm (Glcβ1) with a sphingosine base and nFA [44]. These values were also practically identical to the ones found in porcine small intestine.

Lactose inhibition

Competition experiments were performed to assess whether lactose could inhibit the binding of *P. gingivalis* to cerebroside fractions, sulfatide fractions and a lactosylceramide/galabiosylceramide-containing fraction. The bacterial solution was preincubated with free lactose before bacterial overlays assays was performed as described in "Materials and methods" [55]. Incubation with lactose (0.05, 0.1, and 0.2 mg/ml in PBS) inhibited bacterial binding to the sulfatide structures (Fig. 8, lanes 3 and 4), but surprisingly neither to lactosylceramide/galabiosylceramide (lane 5) nor to the nonsulfated cerebrosides.

Discussion

Periodontal disease has a multifactorial etiology and it is one of the most common plagues of mankind. The direct causal association of bacterial colonization on the tooth surface and the inflammatory destruction of tooth attachment have been clearly demonstrated. The complexes of microorganisms involved in the process appear to relate to the severity of periodontal destruction [51]. Previous studies have shown that the oral, anaerobic pathogen *P. gingivalis* plays a significant role in the development of periodontitis. This specific microorganism seems to act as a key bacterium that is recovered among a majority of the microbial complexes causing severe and aggressive periodontitis with a resulting loss of periodontal attachment. As previously mentioned, an adhesin of the bacterium has been properly examined by among others Dickinson *et al*. [13] and Lee *et al*. [33], but potential receptor structure(s) has, until now, not been characterized. *P. gingivalis* uses the adhesin on the tip of its fimbriae to get close to the host epithelium. The binding can be abolished by blocking the

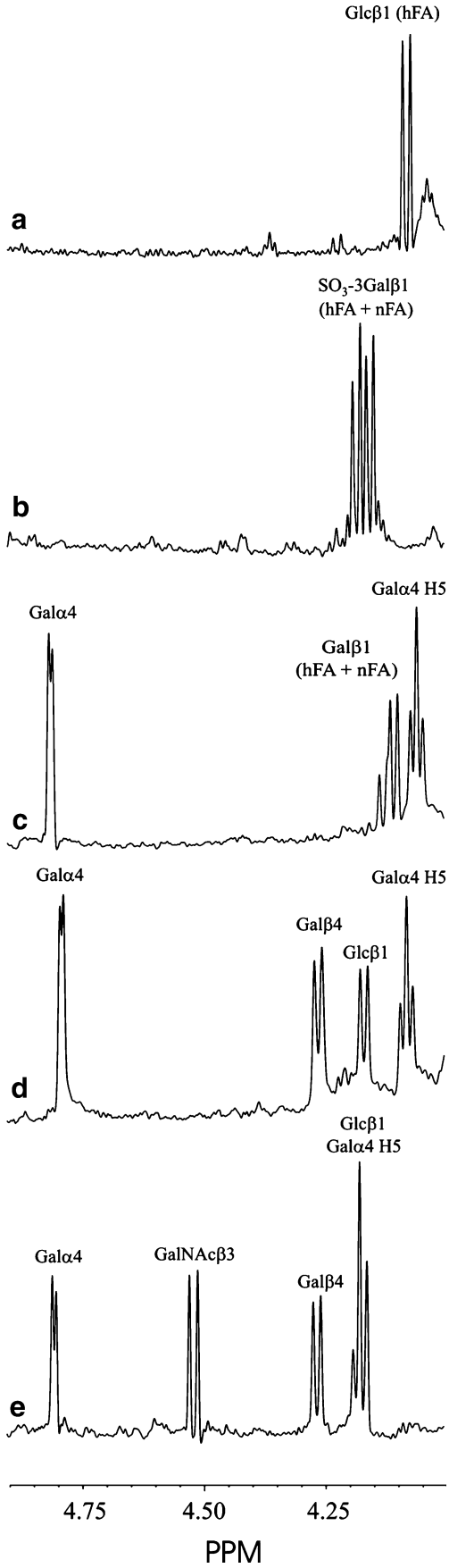

Fig. 7 The anomeric regions of selected ^{1}H NMR spectra from the structures listed in Table 2 are shown. **a** Glucosylceramide (*Glcβ1Cer*) with hFA from porcine spleen, **b** sulfatide (*SO$_3$-3Galβ1Cer*) with hFA and nFA from human kidney, **c** galabiosylceramide (*Galα4Galβ1Cer*) with hFA and nFA from porcine small intestine, **d** globotriaosylceramide (*Galα4Galβ4Glcβ1Cer*) from porcine small intestine, and **e** globotetraosylceramide (*GalNAcβ3Galα4Galβ4Glcβ1Cer*) from porcine small intestine. Anomeric resonances are only labeled by their sugar origin whereas Galα4 H5 resonances are indicated as such

fimbriae by monoclonal antibodies [26]. *P. gingivalis* expressing a major arsenal of fimbriae shows an increased binding capacity compared to strains expressing only a minor arsenal to the same epithelial cells [34].

The FimA gene coding for the fimbriae protein fimbrillin has been isolated [13, 33]. Bacterial fimbrillin amino acid sequence peptides have been synthesized and used to abolish bacterial adhesion in *e.g.* the case of saliva-coated hydroxyl apatite [34].

P. gingivalis express *in vivo* HA-Ag2, a haemagglutinating adhesin [40] presumably connected to the subunits of *P. gingivalis* fimbriae [10]. Laboratory isolates of the bacteria but also clinical strains have been shown to invade both laboratory cell lines [11, 46] and primary cell cultures isolated from oral epithelium [47]. The fimbriae adhesin is probably essential for the bacterial adhesion to the epithelial cell (Sandros, unpublished results). Some bacterial strains has been shown to lack fimbriae due to mutations in the

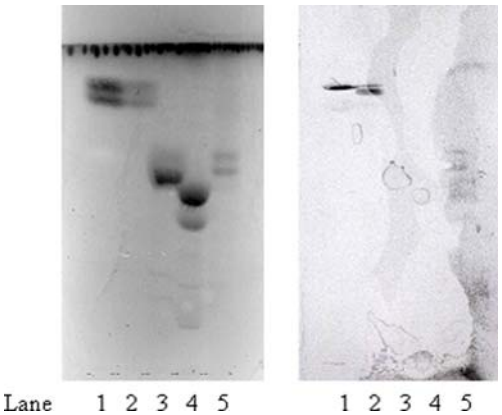

Fig. 8 Thin layer chromatogram of inhibition experiments with lactose. **a** Detection of glycolipids with anisaldehyde reagent. **b** Binding pattern of the bacteria visualized by autoradiography. ^{35}S-labeled *Porphyromonas gingivalis* were preincubated for 1 h with lactose in PBS (0.2 mg/ml), thereafter the binding assay was performed as described in "Materials and methods." 4–8 μg of the glycolipids were added to the lanes (*1–5*). The solvent system was chloroform/methanol/water (60:35:8, by volume). The lanes contained a mixture of Galβ1Cer and Glcβ1Cer from porcine salivary gland (*lane 1*), Galβ1Cer from porcine kidney (*lane 2*), SO$_3$-3Galβ1Cer from human kidney (*lane 3*), SO$_3$-3Galβ4Glcβ1Cer from human kidney (*lane 4*), and Galβ4Glcβ1Cer/Galα4Galβ1Cer from porcine kidney (*lane 5*)

Table 3 Summary of putative glycolipid binding epitopes for *Porphyromonas gingivalis*

Structures	Sequence[a]	Comment
Cerebrosides	*Glcβ1*Cer	Ceramide involvement
	*Galβ1*Cer	
Lactosylceramide	*Galβ4Glcβ1*Cer	Ceramide involvement
Sulfatides	*SO_3-3Gal*β1Cer	
	*SO_3-3Gal*β4 Glcβ1Cer	
Globo	*Galα4 Gal*β1Cer	
	*Galα4Gal*β4 Glcβ1Cer	
	GalNAc β3*Galα4Gal*β4 Glcβ1Cer	
Ganglio	*Galβ3GalNAc*β4Galβ4Glcβ1Cer	
Lacto	*Galβ3GlcNAc*β3Galβ4Glcβ1Cer	
Neolacto	*Galβ4GlcNAc*β3Galβ4Glcβ1Cer	

[a] Putative binding epitopes of the different structures are shown in italics.

FimA gene, as for example the DPG3 strain. It is almost impossible for this strain to invade tissue (Sandros, unpublished results) in contrast to. *P. gingivalis* with normal fimbriae expression, which quickly penetrate the cell layer in pocket epithelium *in vitro* [43]. This implies that the bacterium can elude the human response of epithelium shedding as a defense for infection.

In this study we have focused on the strain FDC 381, a laboratory strain often used in *in vitro* experiments. Though a variety of strains exists, it is important to stress the question if the binding pattern to glycoconjugates will vary between these different clinical and laboratory strains of *P. gingivalis* with different phenotypes, different cell binding properties and invading capacity.

Bacterial adhesion of *e.g. E. coli, H. pylori* and *S. suis* to short carbohydrate sequences has been confirmed in previous studies [29]. This binding process has been thoroughly studied at different locations, such as the human intestine [9, 21] and the oral cavity [53]. Both nonacid and acid carbohydrate chains are known to act as bacterial receptors [3, 9, 21, 29, 37, 55, 56]. In our previous study we showed for the first time that also the *P. gingivalis* strain FDC381 can utilize glycolipid sugar chains as potential bacterial receptors [20].

The bacterial overlay assays showed that *P. gingivalis* favored adherence mainly to short glycolipid structures containing one to four sugar residues as shown in Table 1 and proposed binding epitopes have been summarized in Table 3. Starting with the cerebroside structures (Galβ1Cer and Glcβ1Cer) it was found, in contrast to bacteria adhering in the gastrointestinal tract, that *P. gingivalis* preferred ceramides with nonhydroxylated fatty acids. Based on these results it is most likely that the bacterium does not distinguish between Gal and Glc residues, indicating that the sugar ring 4-OH group is unimportant. Binding to lactosylceramide (Galβ4Glcβ1Cer), on the other hand, requires hydroxylated fatty acids in the ceramide, suggesting a separate binding epitope that also includes isoglobotriaosylceramide (Galα3Galβ4Glcβ1Cer), since the Galα does not affect the bacterial binding. However, in both cases the lactose failed to inhibit binding, which may be taken to indicate that the ceramide head is involved in recognition of these two binding epitopes either directly or by influencing the epitope presentation [1, 3]. Binding to sulfatide (SO_3-3Galβ1Cer) and sulfated lactosylceramide (SO_3-3Galβ4Glcβ1Cer) involves only the terminal 3-sulfated galactose since the presence of Glc in the latter structure does not affect binding. This conclusion is supported by the fact that binding is independent of the ceramide composition and that it is able to inhibit binding. In analogy *H. pylori* also appears to recognize both sulfatide [45] and sulfated lactosylceramide under some conditions [49], but not others [3]. One might expect, however, that 3-sulfated gangliotetraosylceramide (SO_3-3Galβ3GalNAcβ4Galβ4Glcβ1Cer) would be binding positively, but in this case the binding epitope presentation is expected to be very dissimilar to those of the shorter glycolipids and/or that the penultimate GalNAc might influence binding negatively.

Galabiosylceramide, globotriaosylceramide and globotetraosylceramide (Galα4Galβ1Cer, Galα4Galβ4Glcβ1Cer and GalNAcβ3Galα4Galβ4Glcβ1Cer) form yet another group of glycolipids, having in common the Galα4Gal disaccharide, which most likely constitutes a binding epitope similar to the one utilized by uropathogenic *E. coli* [8].

The last three binding epitopes can be treated collectively since they all utilize a terminal LacNAc sequence. Neolactotetraosylceramide (Galβ4GlcNAcβ3Galβ4Glcβ1Cer) thus reveals an absolute dependence on the Galβ4Glc NAcβ3 sequence since de-*N*-acylation abolishes binding as found previously for *e.g. H. pylori* [38]. Secondly, both gangliotriaosylceramide and gangliotetraosylceramide (Gal NAcβ4Galβ4Glcβ1Cer and Galβ3GalNAcβ4Galβ4Glc β1Cer) are binding positive suggesting that the Galβ3Gal NAcβ4 sequence constitutes the binding epitope in analogy with *H. pylori* [3] and *H. influenzae* and *N. meningitidis* [24]. The requirement for the terminal Galβ3 is evident from the negative binding of sulfated gangliotetraosylceramide (SO_3-3Galβ3GalNAcβ4Galβ4Glcβ1Cer). Thirdly, lactotetraosylceramide (Galβ3GlcNAcβ3Galβ4Glcβ1Cer) is binding positively as found also in the *H. pylori* [55] and *H. influenzae* and *N. meningitidis* [24] cases. Although comparative data from isostructures are lacking, in this case the analogy with other bacteria is strongly suggestive of a Galβ3GlcNAcβ3 binding epitope. It should be stressed, however, that the binding epitopes just described, even

though all three contain a LacNAc segment, are topographically distinct and would thus require separate adhesins for binding to occur.

Although several binding structures for *P. gingivalis* and their putative binding epitopes have been identified a number of questions still remain to be resolved, among them which of these structures are present in the periodontal epithelium and whether they are cryptitopic or not. Due to very limited access, preparation and structural analysis of glycolipids present in periodontal tissue is a prohibitive undertaking, but mass spectrometric analyses of ceramidase-treated glycolipids, releasing the oligosaccharides, might be a practicable route forward requiring amounts only in the femtomol range.

Concluding remarks

Binding of *P. gingivalis* to carbohydrate epitopes might act as a first step in the invasion of pocket epithelial cells, a process that subsequently leads to damage of the periodontal tissue. In cases with very advanced or rapidly progressing periodontitis the use of antibiotics may be successful. However, such treatment is associated with a risk of developing antibiotic-resistant bacterial strains and therefore an alternative method of therapy is required. A structural characterization of the bacterial binding epitopes is an essential first step in the process of finding alternative methods of treatment involving the use of receptor analogues in anti-adhesion therapies or vaccines specifically directed against *P. gingivalis* infection. The future goal is to reduce incidence, onset, and spread of periodontal disease.

Materials and methods

Sample criteria

A summary of all glycolipid fractions that were used in this study is shown in Table 1. They mainly consist of total glycolipid fractions, which tested positively for bacterial binding [20] complemented with specific pure reference glycolipid fractions.

High performance liquid chromatography

The total glycolipid fractions were further purified and fractionated by silicic acid column chromatography on a HPLC system using a linear gradient of chloroform/methanol/water (80:20:1 or 65:25:4 to 40:40:12 by volume). A constant flow of 2 ml/min was used over 280 min. Fractions of 4 ml were collected and analyzed by thin-layer chromatography and anisaldehyde staining. Thereafter, the fractions were pooled according to the mobility in the thin-layer chromatograms and used in bacterial overlay assays with ^{35}S-labeled *P. gingivalis*.

Thin layer chromatography

TLC was performed as previously described [16] on aluminum-backed silica gel 60 high performance thin layer chromatography plates (Merck, Darmstadt, Germany and HP-KF, Whatman, Maidstone, UK). Glycolipid fractions (2–40 μg/lane) were added to the TLC plates, after which the nonacid components were chromatographed in chloroform/methanol/water (60:35:8 by volume) and the acid components in chloroform/methanol/water with 0.2% $CaCl_2$ (60:40:9 by volume). The TLC plates that were to be used in the bacterial overlay assays were coated in either 0.3% (*w*/*v*) polyisobutylmethacrylate, P28 (Sigma-Aldrich, Stockholm, Sweden) in diethyl ether/*n*-hexane (1:1 by volume), or in 0.5% P28 in diethyl ether/*n*-hexane (1:4 by volume) and left to polymerize overnight to avoid unspecific binding to non-polymerized monomers. All glycolipid-containing lanes were visualized by chemical detection with anisaldehyde reagent [28].

Desialylation and de-*N*-acylation

Sialyl-neolactotetraosylceramide (1.5 mg) was desialylated by incubation with 1.5% CH_3COOH for 3 h at 100°C. Methanol was added to the sample followed by evaporation using nitrogen at 40°C. This procedure was repeated three to four times until the sample was completely dry. Thereafter, the sample was resuspended in chloroform/methanol (2:1 by volume) and treated as described in the following section.

The GlcNAcβ3 residue in neolactotetraosylceramide was selectively de-*N*-acylated by treatment with anhydrous hydrazine as described by Ångström *et al*. [3]. Two hundred micrograms of the glycolipid was dissolved in 300 μl anhydrous hydrazine (BioRad, Hercules, USA), sonicated for 30 s, where after the reaction was allowed to proceed for 72 h at 76°C. The hydrazine was subsequently removed using nitrogen at 40°C, followed by two cycles of re-dissolution in toluene and evaporation prior to redissolution of the residue in approximately 100 μl methanol with the aid of sonication. After addition of 10 ml water the solution was passed through a 500 mg C18 column (Amprep™Octadecyl C18 Minicolumn, GE Healthcare, Uppsala, Sweden) pre-washed in 5 ml chloroform, 5 ml chloroform/methanol (2:1), and 2 ml water. The sample was eluated with 5 ml methanol, 5 ml chloroform/methanol (2:1), and 5 ml chloroform/methanol/water (40:40:12). Thereafter, the sample was evaporated with nitrogen at 40°C and used in a bacterial overlay assay.

Bacterial strains and growth conditions

P. gingivalis FDC381 (collection of Forsyth Dental Center, Boston, USA), a type strain isolated from a patient with periodontal disease, were used in all experiments. The bacteria were grown on Brucella agar plates (BBL Microbiology Systems, Cockeysville, USA.) enriched with 5% defibrinated horse blood, 0.5% hemolyzed blood and 5 μl/ml menadione in jars with 95% H_2 and 5% CO_2 at 37°C. A suspension of 5 μl L-[^{35}S]Methionine and L-[^{35}S]Cysteine in phosphate-buffered saline (PBS) with a concentration of 14.3 mCi/ml and $t_{1/2}$ of 87.4 days (Redivue ProMix L-[^{35}S] *in vitro* labeling mix, Amersham Biosciences, Uppsala, Sweden) was added to the agar plates. After 3 days of growth, bacterial cultures were collected and washed twice by centrifugation at approximately 5,000 rpm in PBS solution. A spectrophotometer analysis at 550 nm was performed with PBS as standard for evaluation of the bacterial concentration. The bacteria were suspended in PBS with 2% bovine serum albumin (BSA) (*w*/*v*), 0.1% Tween 20 (*v*/*v*) and diluted to a final concentration of 1.0×10^8 bacteria/ml used in the bacterial overlay assays.

Bacterial overlay and detection of bacterial binding

The method used in the bacterial overlay assays is a modified version of the method by Karlsson and Strömberg [30]. The TLC plates were coated with 2% BSA (*w*/*v*) and 0.1% Tween 20 (*v*/*v*) in PBS for 2 h at room temperature to block nonspecific binding sites. Thereafter, approximately 5 ml of the bacterial suspension was sprinkled over the TLC plates followed by overnight incubation at room temperature. The incubation was terminated by gentle washing with PBS solution. The plates were dried at least 1 h at room temperature, and autoradiographed for approximately 7–14 days using a β-sensitive film (Kodak BioMax MR, Amersham Biosciences, Uppsala, Sweden). Bacterial binding was assessed by visual inspection and significant staining/darkening of the film was registered as positive binding.

Inhibition assay with lactose

To test if the binding could be inhibited by soluble sugars ^{35}S-labeled *P. gingivalis* were incubated for 1 h with various concentrations (0.05, 0.1, and 0.2 mg/ml) of lactose in PBS [55]. Thereafter, the bacterial overlay assay was performed as previously described.

Proton nuclear magnetic resonance spectroscopy

^{1}H NMR spectra were acquired on a Varian 500 MHz spectrometer at 30°C. Prior to analysis by NMR the samples were dissolved in dimethylsulfoxide-d_6 and D_2O (98:2 by volume) after deuterium exchange.

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