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Studies on the binding of bacteria to glycolipids

Two species of *Propionibacterium* apparently recognize separate epitopes on lactose of lactosylceramide

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Two species of *Propionibacterium* were analysed regarding their binding to glycosphingolipids. Bacteria were labeled with ^{125}I and selective interaction with glycolipids on thin-layer chromatograms was revealed by autoradiography. The carbohydrate site in common for active molecular species appeared to be lactose. The two bacteria differed, however, in the overall binding pattern on the chromatogram, probably due to recognition of separate epitopes on lactose. *P. freudenreichii* bound only to lactosylceramide while *P. granulosum* also recognized substituted lactosylceramide: Galα1→3Galβ1→4GlcβCer, GlcNAcβ1→3Galβ1→4GlcβCer and Galβ1→3GlcNAcβ1→3Galβ1→4GlcβCer were active, but Galα1→4Galβ1→4GlcβCer was inactive. Also, there was an interesting dependence on ceramide structure in the case of lactosylceramide. *P. freudenreichii* bound to lactosylceramide with sphingosine and non-hydroxy fatty acids but not to species with sphingosine and 2-hydroxy fatty acids, phytosphingosine and non-hydroxy fatty acids or phytosphingosine and 2-hydroxy fatty acids. For *P. granulosum* the situation was reversed. This may be explained by an influence of ceramide structure on the presentation of the two lactose epitopes at the assay surface. These results were supported by curves from the binding of labeled bacteria to glycolipids coated in microtiter wells and in part by binding to glycolipid-coated chicken erythrocytes.

Bacterial adhesion; Receptor; Glycolipid; Lactosylceramide; (*Propionibacterium*)

1. INTRODUCTION

Adherence of bacteria to host tissue surfaces has been considered an important initial event in colonization and pathogenesis [1,2]. Cell surface carbohydrates (i.e. glycolipids and glycoproteins) have long been implicated as receptors in this process [1,2]. However, only a few carbohydrate receptors have been defined in some detail (e.g. [3,4]) and in many cases the only evidence is inhibition of binding by simple saccharides or treatment of cells with carbohydrate-destroying reagents such as hydrolases and periodate [1,2]. We have recently developed a technique to test directly the ability of glycolipids separated on a thin-layer plate to mediate specific attachment of bacteria and viruses [5–7]. Upon screening of microbiological ligands using this approach several novel carbohydrate specificities were revealed [5–9] and are now being characterized [10,11]. Among these a large number of commensal and pathogenic bacteria were found to bind specifically to lactosylceramide [9].

Here, we describe two species of *Propionibacterium* which apparently recognize different epitopes on lactosylceramide (LacCer). Also, there was an interesting dependence on ceramide structure for accessibility of these epitopes.

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2. MATERIALS AND METHODS

2.1. *Bacteria and labeling*

P. freudenreichii (strain ATCC 6207), isolated from dairy products, and *P. granulosum* (strain ATCC 25564), isolated from human skin, were grown for 18 h on Todd-Hewitt agar supplemented with 5% (v/v) sheep blood, harvested and washed in phosphate-buffered saline (PBS), pH 7.3. After two cycles of washing and pelleting in a refrigerated centrifuge (4000 × *g*, 5 min, 4°C) approx. 2×10^8 bacteria of each strain were externally labeled with ^{125}I using 100 μCi Bolton-Hunter reagent [12] delivered by New England Nuclear (Boston, MA). However, the batch of *P. granulosum* used for fig.2 was ^{125}I labeled using the solid-phase reagent tetrachlorodiphenylglycoluril (Iodogen, Pierce, Rockford, IL) as described in [6]. Labeled bacteria were pelleted in a table centrifuge, washed twice in PBS, and finally resuspended in PBS to give suitable radioactivity counts (see figures).

2.2. *Glycolipids*

Preparation of total non-acid glycolipids of various sources was carried out as in [13]. The composition of the fractions used for fig.1 has been reported for human erythrocytes [14,15], human meconium [16], dog small intestine [17], rabbit small intestine [18], guinea pig small intestine [19] and rat small intestine [20]. The chemical structures of purified compounds (table 1) were confirmed by mass spectrometry, NMR spectroscopy and degradation, in principle as described [14–20]. The preparation and structural characterization of lactosylceramides with different ceramide composition will be described in detail elsewhere.

2.3. *Thin-layer chromatography*

Thin-layer chromatograms were developed on aluminum sheets coated with silica gel 60 (HPTLC nano plates, Merck, FRG) using chloroform/methanol/water (60:35:8, by vol.) as solvent. The anisaldehyde reagent [21] was used for chemical detection.

2.4. *Binding assays*

Binding of radiolabeled bacteria to glycolipids separated on thin-layer plates was according to [6,7]. Briefly, two chromatograms were developed in parallel on the same sheet. After cutting, one was sprayed with anisaldehyde and the other was treated with 0.5% (w/v) polyisobutylmethacrylate in diethyl ether followed by blocking of unspecific sites with 2% (w/v) bovine serum albumin in PBS. After incubation with labeled bacteria, the chromatogram was repeatedly washed and finally autoradiographed. Binding of ^{125}I-labeled bacteria to serial dilutions of glycolipids coated in polyvinyl chloride microtiter wells was performed as in [7]. More details are given in the figure legends.

Chicken erythrocytes coated with purified glycolipids were tested for binding of radiolabeled bacteria. The coating was done by incubation of erythrocytes (3% in PBS) with glycolipids (1 mg/ml) for 2 h at 37°C. After washing, erythrocytes were incubated with ^{125}I-labeled bacteria for 1 h at 25°C. The assay mixture was then layered on a discontinuous Percoll (Pharmacia, Uppsala) gradient (0–20–100%) and centrifuged for 12 min at 800 × *g* at 25°C. The erythrocytes layered at the 20–100% Percoll interface. The top Percoll layers containing unbound bacteria were removed with a pasteur pipette. Both fractions were then counted in a gamma counter.

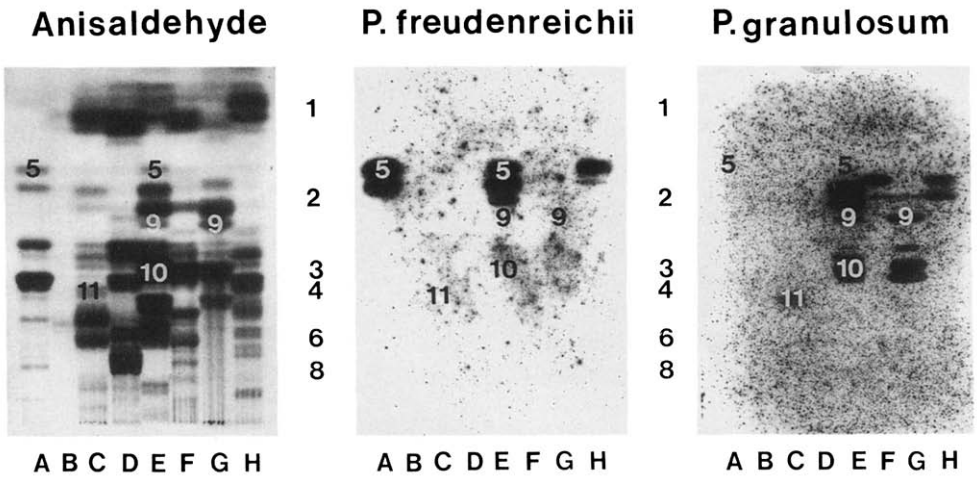

Fig.1. Thin-layer chromatograms of separated glycosphingolipids detected with anisaldehyde (left) or autoradiography after binding of ^{125}I-labeled *P. freudenreichii* (middle) and *P. granulosum* (right). Total non-acid glycolipids (10–40 μg of each lane) of the following sources were applied (lanes): human erythrocyte of blood group A (A), human meconium of blood group A (B) and H (C), monkey intestine (D), dog small intestine (E), rabbit small intestine (F), guinea pig small intestine (G) and rat small intestine (H). Vertically placed figures indicate the number of sugars for glycolipids with corresponding mobility. The identity of several of the bands is given by a number referring to table 1. Incubation was for 2 h with radiolabeled *P. freudenreichii* (10^8 cells/ml, 4.5×10^6 cpm/ml) and *P. granulosum* (10^8 cells/ml, 4×10^6 cpm/ml), respectively. After 5 washings the plates were autoradiographed for 80 h.

3. RESULTS

P. freudenreichii and *P. granulosum* gave distinctly different autoradiograms when exposed to the same mixtures of glycolipids (fig.1). However, the interpretation was that both bacteria recognized lactose but with slightly different binding epitopes. The two species had different saccharide preferences as well as a dependency of binding on separate molecular species of ceramide.

From the data in table 1 both bacteria were concluded to have lactose (Galβ1⟶4Glc) as the common and minimal binding saccharide. However, while *P. freudenreichii* was able to bind to LacCer only, *P. granulosum* also bound to some 3- and 4-sugar glycolipids, indicating recognition of an internally placed sequence, as documented earlier for *E. coli* [4] and the Shiga toxin [22,23]. Substitutions of lactose with Galα1⟶3 (no. 10) or GlcNAcβ1⟶3 (no. 11) were tolerated, but Galα1⟶4 (nos 12–14) or longer sequences (cf. nos 11 and 15) blocked the binding, probably for steric reasons.

Interestingly, the autoradiograms also differed in the 2-sugar LacCer region (fig.1). On closer analysis, using purified molecular species of LacCer with different chromatographic mobilities (to be described elsewhere), it was found that *P. freudenreichii* recognized species with sphingosine and non-hydroxy fatty acids only (faster-moving). In contrast, *P. granulosum* was not able to bind to these species but required sphingosine and 2-hydroxy fatty acids, or phytosphingosine and non-hydroxy fatty acids, or phytosphingosine and 2-hydroxy fatty acids (slower-moving). This means that *P. granulosum* did not bind to LacCer of human erythrocytes (no. 5 of lane A), which practically lacks more hydroxylated ceramides. There

Table 1

Characteristics of binding of *P. freudenreichii* and *P. granulosum* to glycolipids[a]

No.[b]	Glycolipid[c]	Lipophilic part[d]	Binding[e] of	
			P. freudenreichii	*P. granulosum*
1	Galβ→Cer	varying ceramide components	–	–
2	Glcβ→Cer	varying ceramide components	–	–
3	Galα1→4Galβ→Cer	varying ceramide components[f]	–	–
4	Galβ1→4Glcβ→Cer	$OCH_2CH_2S(CH_2)_nCH_3$[g]	+	–
5	Galβ1→4Glcβ→Cer	d18:1-16:0/24:1[h]	+	–
6	Galβ1→4Glcβ→Cer	varying ceramide components[i]	+	+
7	Galβ1→4Glcβ→Cer	d18:1-h24:0[i]	not tested	+
8	Galβ1→4Glcβ→Cer	t18:0-24:0[i]	not tested	+
9	Galβ1→4Glcβ→Cer	t18:0-h24:0[i]	–	+
10	Galα1→3Galβ1→4Glcβ→Cer	d18:1/t18:0-h16:0/24:0[i]	–	+
11	Galβ1→3GlcNAcβ1→3Galβ1→4Glcβ→Cer	d18:1/t18:0-h24:0[k]	–	+
12	Galα1→4Galβ1→4Glcβ→Cer	d18:1-16:0/24:1	–	–
13	GalNAcβ1→3Galα1→4Galβ1→4Glcβ→Cer	d18:1-24:1[h]	–	–
14	GalNAcα1→3GalNAcβ1→3Galα1→4Galβ1→4Glcβ→Cer	d18:1-16:0/24:0[i]	–	–
15	Fucα1→2Galβ1→3GlcNAcβ1→3Galβ1→4Glcβ→Cer	d18:1/t18:0-h16:0/h24:0[k]	–	–

[a] Assessment for qualitative binding was performed with the chromatogram binding assay, as described in fig.1 and section 2

[b] Numbers refer to the figures and the text

[c] Glycolipid nomenclature is according to ICBN, Eur. J. Biochem. (1977) 79, 11–21 and J. Biol. Chem. (1982) 257, 3347–3351

[d] Shorthand designations are according to an earlier recommendation [30]. Thus, d denotes dihydroxy base, t trihydroxy base, and h 2-hydroxy fatty acid. No prefix signifies non-hydroxy fatty acid. The figures before a colon represent paraffin chain length and after the colon the number of double bonds. The term varying components means a mixture of dihydroxy and trihydroxy bases and hydroxy and nonhydroxy fatty acids

[e] +, binding; –, nonbinding; at 1 μg glycolipid

[f] Colon cancer tissue

[g] Purchased from the Swedish Sugar Co. (Arlöw, Sweden)

[h] Human erythrocyte

[i] Whole dog small intestine

[k] Human meconium

was, however, no strict specificity for the ceramide as either phytosphingosine or 2-hydroxy fatty acids fulfilled the requirement for *P. granulosum*, and ceramide could be replaced by a single lipophilic chain (no. 4) in the case of *P. freudenreichii*. This dependency on ceramide structure is restricted to free LacCer and does not appear for glycolipids with longer saccharide chains (to be published).

Binding of the two ^{125}I-labeled *Propionibacterium* species to varying amounts of selected glycolipids coated in microtiter wells supported the existence of two separate binding specificities (fig.2). Accordingly, *P. freudenreichii* attached to LacCer with dihydroxy base and non-hydroxy fatty acids (no.5) in a fairly small amount (half-maximal binding at 50 ng), whereas *P. granulosum* failed to interact with this compound. As expected, both bacteria bound LacCer isolated from dog intestine (no. 6), which has a heterogeneous ceramide (cf. lanes A and E in fig.1).

In accordance with the above studies using solid-phase glycolipids, chicken erythrocytes coated with mixed LacCer of dog intestinal origin (no. 6) bound 7-times more *P. granulosum* than both uncoated cells or those coated with LacCer of human erythrocytes (no. 5). For *P. freudenreichii* cells coated with these LacCer samples (nos 5,6) bound 3–4-times more bacteria compared to uncoated cells.

4. DISCUSSION

Our results on bacterial binding to glycolipids immobilized on various surfaces indicate that *P. freudenreichii* and *P. granulosum* recognize different portions on lactose (Galβ1⟶4Glc) of LacCer (figs 1,2 and table 1).

Firstly, the two bacteria had separate preferences concerning saccharides with more than two sugars (substitutions on lactose). *P. freudenreichii* bound to free LacCer only while *P. granulosum* also recognized lactose as an internal sequence. Thus, Galα1⟶3 or GlcNAβ1⟶3 added to lactose were tolerated but Galα1⟶4 was not. The dissimilarity of these substitutions makes it unlikely that they are included in the true recognition site of the presumed lectin. Other microbiological ligands are also able to recognize internally located sugar sequences, including uropathogenic *E. coli* [4] and the Shiga toxin [22,23], both with specificity for Galα1⟶4Gal. A

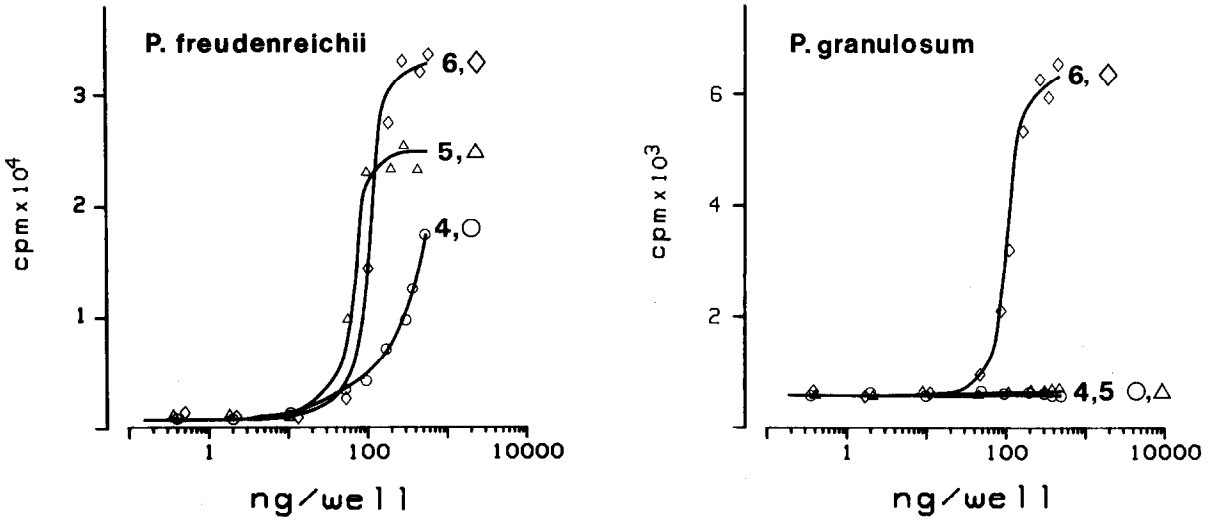

Fig.2. Binding of ^{125}I-labeled *P. freudenreichii* (upper) and *P. granulosum* (lower) to selected glycolipids coated in microtiter wells as in [7]. Numbers to the right of each curve refer to table 1. Incubation for 4 h with *P. freudenreichii* (10^8 cells/ml, 4.5×10^6 cpm/ml, 50 μl/well) and *P. granulosum* (10^8 cells/ml, 4.5×10^6 cpm/ml, 50 μl/well). After 5 washings with PBS, the wells were dried, cut, and measured for radioactivity in a γ-counter. Data are expressed as means of triplicate determinations with standard deviations less than 10%.

more detailed discussion of the binding specificity of *P. granulosum* will be published elsewhere.

Secondly, the ceramide composition influenced the binding. Since a strict stereochemical requirement was absent (the hydroxyl group of phytosphingosine and that of the hydroxy fatty acid were equivalent, see table 1) the most probable explanation is that two separate lactose epitopes are differently presented and available on the assay surface as determined by the ceramide composition. The biological relevance of this dependency on ceramide remains to be shown. However, conformational analysis of hexosylceramides has shown a correlation between the ceramide structure and the orientation of the carbohydrate moiety. Thus, X-ray crystallography of galactosylceramide with sphingosine and 2-hydroxystearic acid demonstrated a shovel-like overall shape [24], whereas an NMR study of glucosylceramide with sphingosine and palmitic acid proposed a straighter conformation [25]. Furthermore, studies with anti-glycolipid antibodies and binding to intact cells [26] or well-defined liposomes [27] support the idea that the ceramide structure may direct the sugar head group conformation.

Attempts to demonstrate further the lactose specificity of these interactions by inhibition of attachment by preincubation of bacteria with free lactose (20 mg/ml) have not been successful. A possible explanation is low-affinity binding as recently demonstrated for the binding of Shiga toxin to Galα1$\longrightarrow$4Gal-containing glycolipids [23]. This is also indicated by the relatively large amounts of glycolipids required for solid-phase binding (fig.2) compared to other systems [4,7].

One may assume that closely related but not identical lectin-like proteins on the bacterial surface underlie these two specificities. The situation may therefore be analogous to the receptor-binding variants of influenza virus, which recognize NeuAcα2$\longrightarrow$3 and NeuAcα2$\longrightarrow$6, respectively, as a consequence of a single amino acid substitution in the binding site of the hemagglutinin [28].

The two bacteria analysed were of different origin, *P. granulosum* being isolated from human skin whereas *P. freudenreichii* was isolated from milk products. It is of interest that the ceramide variants being differentially recognized by the two bacteria show a distinct tissue localization in the human: the more hydroxylated ceramide (phytosphingosine and/or hydroxy fatty acids) is typical of epithelial cells, while the less hydroxylated species (sphingosine and non-hydroxy fatty acids) exist in non-epithelial cells [29]. Hypothetically, a bacterium may therefore shift target cells by a limited mutation of its lectin.

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