ELSEVIER

Biochimica et Biophysica Acta 1455 (1999) 375–386

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

www.elsevier.com/locate/bba

Review

Glycolipid receptors for verotoxin and *Helicobacter pylori*: role in pathology

Clifford A. Lingwood *

Departments of Laboratory Medicine and Pathobiology and Biochemistry, University of Toronto, and Research Institute, Hospital for Sick Children, 555 University Avenue, Toronto, ON M5G 1X8, Canada

Received 13 October 1998; received in revised form 1 April 1999; accepted 1 April 1999

Abstract

Eukaryotic cell surface glycolipids can act as both the primary interface between bacteria and their host and secondly as a targeting mechanism for bacterial virulence factors. The former is characterized by redundancy in adhesin–receptor interactions and the latter by a higher affinity, more restrictive glycolipid binding specificity for targeting. Interactions of verotoxin with its glycolipid receptor globotriaosylceramide and *Helicobacter pylori* binding to a variety of different glycolipids, which can be environmentally regulated, provide examples of these differing modes of glycolipid receptor function. Verotoxins are involved in endothelial targeting in the microangiopathies of hemorrhagic colitis and hemolytic uremic syndrome (HUS). The highly restricted binding specificity and crystal structure of the verotoxin B subunit have allowed theoretical modeling of the Gb_3 binding site of the verotoxin B subunit pentamer which provides an approach to intervention. Studies of the role of glycolipid function in verotoxin-induced disease have concentrated on the distribution of Gb_3 and its ability to mediate the internalization of the toxin within the target cell. The distribution of Gb_3 within the renal glomerulus plays a central role in defining the age-related etiology of HUS following gastrointestinal infection with VT producing *Escherichia coli*. *H. pylori*, on the other hand, instigates a less distinct but more complex disseminated gastric inflammation. Studies on the role of glycolipid receptors in *H. pylori* infection have been bogged down in establishing the importance of each binding specificity defined. In addition, the physiological condition of the organism within the various binding assays has not been extensively considered, such that spurious non-physiological interactions may have been elucidated. The identification and cloning of a Le^b binding adhesin and the identification of cell surface hsp70 as a mediator of sulfoglycolipid binding under stress conditions may now allow a more molecular approach to define the role of glycolipid recognition in this infection.

Keywords: Receptor-mediated endocytosis; Cytokine; Glycolipid analogue; Apoptosis

Contents

* Fax: +1-416-813-5993; E-mail: cling@sickkids.on.ca

0925-4439/99/$ – see front matter
PII: S0925-4439(99)00062-9

1. Introduction

My interest in the receptor function of the eukaryotic membrane glycolipids was sparked by the conserved and high level of expression of sulfoglycolipids within the male germ cells of vertebrates and our isolation of a sulfoglycolipid binding protein from testicular germ cells [1] which was later shown capable of inhibiting fertilization in vitro [2] and in vivo [3]. While several hypotheses have been postulated concerning the pivotal role of cell surface carbohydrates in fertilization, even the two most widely accepted have been called into question by studies of transgenic mice [4,5] in which the crucial glycosyl transferase has been knocked out and yet fertility is retained.

It is amazing to realize how little is known with certainty, concerning gamete interaction as compared with other systems of cell recognition. Pathogenic bacterial cell-host attachment provides examples of such systems in which eukaryotic cell surface glycolipids play a major receptor role [6]. Indeed, some bacteria implicated in infertility bind specifically to the sulfoglycolipids characteristic of mammalian spermatozoa [7]. Thus the interaction of bacteria with their host glycolipid receptors may have features in common with sperm/egg binding.

2. Verotoxins

Verotoxins are a family of *Escherichia coli* derived bipartite toxins of the A_1B_5 subunit format. Four members are known VT1, VT2, VT2c and VT2e differing primarily in the B subunit [8]. The latter is the pig edema disease toxin and not found in strains which infect man. The toxins are structurally homologous but VT1 and members of the VT2 series are not cross-neutralizable. VT2 is most often associated with human disease.

Gastrointestinal infection with verotoxin producing *E. coli* (VTEC) strains (primarily serotype 0157:H7) is responsible for two significant pathologies, hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) [9,10]. Both these diseases are microangiopathies of the gastrointestinal and renal glomerular blood vessels, respectively. Of the two, HUS is the more significant disease, being the primary cause of acute pediatric renal failure, and despite aggressive interventional therapy, still shows a morbidity of about 5%.

HC, and particularly HUS, show an age-related incidence, the majority of HUS cases occurring in children under three and in the elderly following gastrointestinal infection with VTEC [11]. HC is usually a prodrome of HUS, likely a result of the closer proximity of the GI blood vasculature to the site of infection. The mechanism of translocation of the toxin across the mucosal barrier is essentially unknown since mucosal cells are toxin receptor negative [12].

HUS is defined by a triad of symptoms: thrombocytopenia, hemolytic anemia and renal microangiopathy. The disease was ascribed to a variety of etiological agents but the pioneering epidemiological studies of Karmali et al. [13] first established a link with gastrointestinal VTEC infection, which has been subsequently confirmed worldwide.

2.1. Role of the glycolipid receptor in disease

Verotoxins (VT1 is virtually identical to the Shiga toxin from *Shigella dysenteriae* and therefore was also called Shiga-like toxin or Stx) bind specifically to the galabiose terminal disaccharide moiety of globotriaosyl ceramide-Gb_3 (Galα1-4–Galβ1-4 glucosyl ceramide) [14–16]. The P1 glycolipid also terminating in a galα1-4 gal disaccharide structure is also bound in in vitro assays. The presence of Gb_3 in the plasma membrane is a prerequisite for cell sensitivity to verotoxin-induced cytopathology in vitro and Gb_3 negative cells can be sensitized to the action of verotoxin by the incorporation of exogenous Gb_3 [17]. However, other cellular processes are necessary for cytopathology, including internalization and intracellular trafficking of the toxin receptor complex. Thus the presence of Gb_3 alone may not necessarily reflect cell selectivity or degree of cell sensitivity to VT [18].

Gb_3 is expressed on human platelets and binds VT [19] but VT has no effect on platelet aggregation in vitro [20] making it unlikely that thrombocytopenia in HUS is directly VT-induced. Similarly, red blood cells contain Gb_3 but bind VT weakly and only at 4°C [21], making it unlikely that VT binding is directly responsible for the hemolysis in HUS either.

Gb_3 expression, however, likely plays a crucial role in determining sensitivity to verotoxin-induced renal disease. HUS is primarily a disease of the renal glomeruli and rarely occurs in adults except in the elderly. Staining of frozen sections of human pediatric kidney shows significant toxin binding to cells within the renal glomeruli [22]. In addition, collecting ducts and a subset of renal tubules are also stained. In the adult, however, there is little or no staining by verotoxin within the glomerulus. A subset of distal tubules and collecting ducts is stained as in the pediatric samples. Thus the age-related incidence of HUS correlates with the expression of the toxin receptor, Gb_3, within the renal glomerulus, thus indicating toxin receptor distribution plays a central role in the induction of pathology. Studies in the pig model of verocytotoxemia support such a conclusion, in that site specific mutagenesis of the VT2e toxin B subunit to alter the glycolipid binding specificity from Gb_4+Gb_3 to Gb_3 alone, resulted in the induction of pathology in different tissues and this correlated with the tissue Gb_3 expression [23].

In the elderly, HUS again is prevalent following gastrointestinal VTEC infection, yet the renal glomeruli of such patients are also receptor negative [22]. The explanation for this finding likely resides in the regulation of Gb_3 synthesis within this tissue. Although renal glomerular endothelial cells from elderly patients are receptor negative in situ, isolation of such glomeruli and culture of the endothelial cells within, results in the growth of endothelial cells which have an extremely high Gb_3 content and are highly sensitive to VT-induced cytopathology in vitro. [24]. Thus, endothelial cells in situ can be induced to synthesize Gb_3 by appropriate stimuli, eg., growth. Similar stimulation of cultured endothelial cell Gb_3 synthesis occurs after treatment with certain cytokines eg. TNF, IL1β or LPS [25–28]. We propose, therefore, that the etiology of HUS in the elderly may be the result of an inappropriate response, as the immune system ages, to circulating LPS from the gastrointestinal infection (or LPS-induced cytokines) to stimulate renal endothelial cell Gb_3 synthesis which sensitizes cells for subsequent verotoxin-induced injury. Thus Gb_3 expression plays a central role in determining the age-related incidence of VTEC-induced HUS.

While the presence of renal glomerular Gb_3 may be a primary risk factor for the development of HUS following gastrointestinal VTEC infection, the regulation of Gb_3 synthesis within the target tissue may be complex. Macrophages and monocytes contain Gb_3 but are insensitive to the cytopathology of verotoxin. Treatment of such cells with VT in vitro results in the increased synthesis of interleukin-1β and tumor necrosis factor, and these are the very cytokines which have been shown to induce Gb_3 synthesis in cultured endothelial cells. [29–31]. Thus, the etiology of HUS may contain an amplification component in which verotoxin itself stimulates the production of cytokines by monocytes which render endothelial cells sensitive to the toxin. Such a scenario might relate to the several days time interval between the onset, and even resolution, of gastrointestinal microvasculopathy as compared with the onset of renal sequelae.

Furthermore, the isoform of Gb_3 synthesized may be highly relevant to determining VT sensitivity. In early studies we showed the importance of the lipid moiety of Gb_3 in VT binding [32,33] and reported

that increasing the fatty acid chain length of Gb_3 increases VT 1 binding, to a C22 limit when further extension becomes inhibitory [34]. In our studies of astrocytoma and ovarian carcinoma cell lines we found differing sensitivities to VT1 despite comparable Gb_3 concentrations. We have found that cells of higher sensitivity tend to synthesize Gb_3 with shorter fatty acid chains (C:16 and C:18) and that the VT–receptor complex is internalized to compartments consistent with the ER, nuclear envelope and nucleus, whereas cells of reduced VT sensitivity synthesize Gb_3 with longer chain fatty acids (C:22 and C:24) and target the receptor complex to organelles consistent with Golgi/endosomes [35]. Thus, intracellular traffic of the toxin–receptor complex may play a role in determining sensitivities to cytopathology and such an intracellular routing may be regulated by the fatty acid isoform of the Gb_3. Apparently VT binding and intracellular trafficking show opposing trends in terms of Gb_3 fatty acid chain length. It is likely that cell surface VT binding is excess to that required for killing and that the routing plays the more significant role in determining cell sensitivity. Epidemiological studies have identified red cell Gb_3 fatty acid content as a risk factor for the development of pediatric HUS following VT exposure [36]. The lack of VT binding to red cells suggests that the red cell glycolipid fatty acid phenotype serves as a marker of the fatty acid content of cells more germane to the disease, e.g., renal endothelial cells, mesangial cells etc.

Recently primary human renal tubular epithelial cells have been shown to be highly susceptible to VT1 in vitro [37]. This correlates with the detection of some tubular damage early in VTEC-induced HUS. However, the overall Gb_3 level did not correlate with increased VT1 cytotoxicity observed following cytokine treatment of such cells, suggesting that Gb_3 isoform and intracellular VT1 trafficking may play a role in modulating epithelial cell VT1 sensitivity.

2.2. Gb_3 and HIV-induced HUS

It is also of interest to note that pediatric AIDS patients have a high incidence of an atypical HUS [38]. Transgenic mouse models of HIV infection have been constructed and one of these shows the spontaneous generation of a nephropathy as a function of age, which is similar to the HUS observed in HIV-infected patients [39]. We have recently found that the renal Gb_3 content of the HIV1 transgenic mouse is markedly elevated compared with wild-type litter mates [40]. Overlay of frozen sections of the mouse transgenic HIV kidney showed increased binding of FITC labeled VT1 and anti Gb_3 to renal tubular cells, suggesting that Gb_3 was elevated in these cells. The results suggest that virus-induced elevation of renal Gb_3 might cause renal nephropathy and may also sensitize HIV patients to VTEC infections.

3. Glycolipid receptor-based approaches to the therapy and prophylaxis of verotoxin-induced disease

3.1. The problem

Since the receptor glycolipid globotriaosylceramide plays such an important role in determining toxin mediated tissue damage, our laboratory has taken the approach of attempting to generate soluble receptor analogues which can bind the toxin and may thereby prevent systemic targeting of Gb_3 containing tissues. This has proved to be a difficult problem and has turned out to have far wider implications in terms of the presentation of membrane glycolipid carbohydrate for ligand binding in general.

While many ligands, particularly of prokaryotic origin, bind glycolipid carbohydrate [6,41] most of the examples studied in detail demonstrate that the carbohydrate sequence is the only determinant of binding specificity. For example, although *E. coli* bearing PapG adhesins bind to globo series glycolipids [42] this binding is effectively inhibited by the galabiose disaccharide, and the same holds for *Streptococcus suis* binding to globo series glycolipids [43]. For both these systems, galabiose derivatives can be used to precisely define the binding epitopes within the saccharide moiety [44,45].

Unfortunately, the situation is quite different for verotoxin recognition of these same glycolipids. While the toxin binding specificity resides exclusively within the carbohydrate moiety of the glycolipid [46], the binding of verotoxin to galabiose containing glycolipids is markedly affected by the lipid moiety

[32,34] such that the binding can be completely ablated if an inappropriate species is conjugated [33]. Similarly the binding to the free oligosaccharide [47] is at least five orders of magnitude reduced compared to Gb_3 within a model membrane [48].

3.2. Gb_3 binding site

Our molecular modeling studies, combined with binding to galabiosyl glycolipid deoxy analogues have identified two potential Gb_3 binding sites per B subunit monomer [46,49,50] (Fig. 1). Binding within site 1, essentially a cleft between adjacent B subunit monomers, is consistent with site specific mutagenesis studies thus far reported and was used in homology modeling to explain the additional binding of VT2e to Gb_4 and why all members of the verotoxin family preferentially bind aminoGb_4 [46]. The second site, a depression in the B subunit surface which opposes the host cell plasma membrane, was postulated to be more important in the binding of VT2c and might possibly explain the different spectrum of pathology in animal models observed for this toxin [48]. The second site accommodated a different Gb_3 carbohydrate conformer in respect to the relative orientation of the glycosidic linkage to the ceramide [46]. Previous theoretical studies [51] had indicated that the relative conformation of the carbohydrate with respect to the plasma membrane was in fact determined by the relative plane of the plasma membrane in relationship to the head group of the glycolipid. This relationship will be determined by the lipid composition of the plasma membrane and the lipid composition of the glycolipid. Thus fatty acid heterogeneity may well influence the relative plane of the plasma membrane in respect to the glycolipid head group and thereby alter the distribution of conformations. Such an effect would then explain the differential binding of VT1 and VT2c to different Gb_3 fatty acid isoforms [34]. Potentially different fatty acid isoforms might preferentially dock into each of the two distinct Gb_3 binding sites within the B subunit.

The verotoxin 1B subunit pentamer has now been cocrystallized with the Gb_3 oligosaccharide linked to a C8 hydrocarbon chain [52]. This study identified three Gb_3 binding sites per monomer, one corresponding to the cleft site, one to the second site, similar to that defined by modeling, and a third corresponding to the region of tryptophan 34 (see Fig. 1). Site specific mutagenesis, however, suggests that this third site plays a minor role in VT cytotoxicity. The orientation of the Gb_3 sugar in site I is similar to that predicted except that the interactions involving the terminal α-galactose are different, such that the sugar is more perpendicular to the cell surface and does not interact with residues from the adjacent subunit (this orientation does not explain the binding

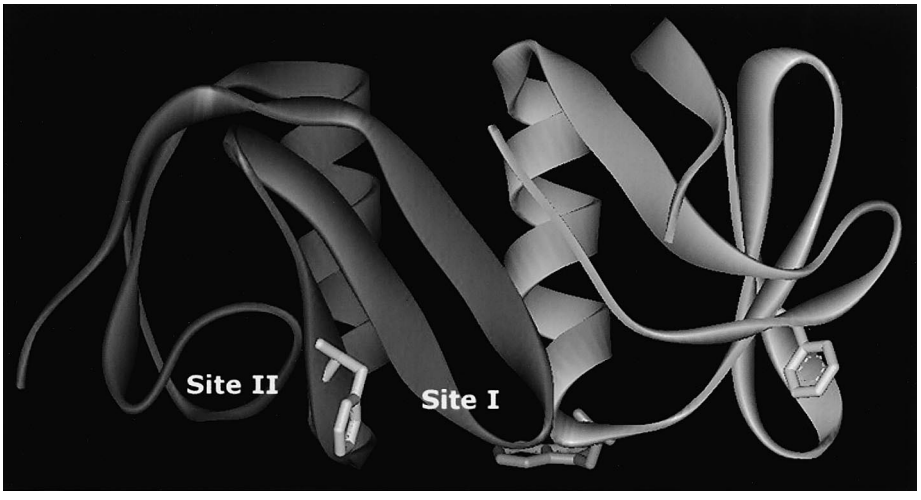

Fig. 1. Location of the putative Gb_3 binding site in the VT B subunit. Two of the five B subunit monomers are shown. The view is from the side – the surface opposing the cell membrane is at the bottom and the A subunit would be at the top. The position of Phe30, which plays a crucial role in binding [1], is shown. Gb_3 binding sites I and II are essentially on either side of Phe30. The position of Trp34 at the base of the α helix which forms the central pore of the B subunit pentamer is also indicated.

of amino Gb_4). The Gb_3 sugar in site II, however, is oriented in a different direction from that predicted. The solution structure of the VT1 B subunit pentamer complexed with the Gb_3 oligosaccharide has also been recently solved by NMR [53]. These studies confirmed the primary occupancy of site II as observed in the cocrystal. The authors estimate a maximum occupancy of 15% for site I, but saw no evidence for association of the sugar with tryp 34.

These data do not necessarily, however, indicate that the modeling predictions are wrong. As indicated above, the lipid-free Gb_3 sugar is many orders of magnitude less effectively bound by VT1. While multivalency, such as may occur for the intact Gb_3, will increase binding affinity to the B subunit pentamer, the cocrystal studies conclude there is no evidence of cooperativity between binding sites [52]. We propose the lipid moiety plays a crucial role in the 'presentation' of the Gb_3 oligosaccharide for binding and the structure of the complex with the free sugar may be of limited relevance to VT–membrane glycolipid recognition. Studies using fluorescence resonance energy transfer (FRET) on the binding of the VTB subunit to liposomes containing a coumarin derivative of Gb_3 have allowed the measurement of the distance between the fluor of the bound receptor and tryp34 [54]. This distance is consistent with the occupancy of site II as modeled [46] but not with the occupancy of any site as defined by the oligosaccharide–VTB complex.

In order to generate potential high affinity inhibitors of VT/Gb_3 binding, the role of the lipid moiety in binding must be of primary consideration. Structures which can be docked into site I as we have defined it, rather than into the sites inferred from the VT/oligosaccharide cocrystals, have the greatest likelihood of efficacy as inhibitors of VT binding to Gb_3 on target cells in vivo.

It is also possible that 'site-selective' inhibitors could be made. The FRET experiments are consistent with site II occupancy rather than site I [54]. On measurement of receptor activity by TLC overlay, the coumarin-Gb_3 was found to bind preferentially, in comparison to Gb_3, to VT2c rather than VT1, suggesting that the coumarin-Gb_3 may be a Gb_3 derivative which binds preferentially to site II (which we have postulated is preferentially used by VT2c [46]). Thus by modifying the lipid moiety to incorporate a coumarin group, a site II-specific Gb_3 derivative may have been generated.

3.3. Strategy for therapeutic approaches

We have therefore begun a systematic study to determine the influence of the lipid moiety on glycolipid receptor function in order to generate water soluble mimics of membrane glycolipid receptors. We have used two separate approaches.

3.3.1. Generation of galabiosyl dimers

In collaboration with Dr. G. Magnusson, University of Lund, we have examined the ability of a large variety of galabiosyl dimers linked by various spacer groups to inhibit the binding of VT1 to Gb_3 in a receptor ELISA. For the most part these compounds have proven inactive. At the moment, no rationale for the distinction between active and inactive galabiose dimers can be discerned. The active dimers are not sufficient to cross-link sites on adjacent B subunits but may be sufficient to cross-link site I and site II. Minor changes in the nature of the chemical cross-linker make a major difference in the ability to inhibit toxin binding. However, the finding that some monomeric species also inhibit, suggests the possibility that the second galabiose may act as the 'aglycone' to properly orient the first galabiose. Trimeric and tetrameric species were not active.

3.3.2. Soluble glycolipid mimics

The second approach is to try to generate soluble glycolipid derivatives which mimic the effect of the lipid moiety and membrane presentation as determinants of the carbohydrate receptor function. To this end we have developed a new procedure for the quantitative cleavage of the sphingosine double bond to generate the 'glycoceramidic acid'. The procedure involves the catalytic oxidation of the double-bond with potassium permanganate in an oxidation-resistant solvent together with a system for regenerating permanganate from manganese dioxide generated during the oxidation reaction. These two additional components prevent the generation of side reactions and precipitants which remove product. We have been able to obtain yields on the order of 90% of a single product corresponding to the ceramidic acid of any given glycolipid [55]. The availability

of this oxidation procedure opens up a new vista for the generation of new bioactive glycolipid derivatives, including the generation of novel soluble Gb_3 derivatives which can compete for membrane bound verotoxin Gb_3 binding [56]. Such compounds may open up a new approach to the prophylaxis of HUS following gastrointestinal VTEC infection.

Clinical trials using a Gb_3 oligosaccharide immobilized on diatomaceous earth and given orally have not proven particularly effective in reducing HUS following gastrointestinal VTEC infection [57]. The availability of soluble receptor analogues capable of competing for systemic toxin binding may well prove a more effective means of preventing the targeting of GI-derived verotoxin to distal Gb_3-containing tissue sites in susceptible hosts. The availability of an insoluble receptor analogue within the GI tract itself may be of limited protective benefit since the role of free toxin within the gastrointestinal lumen in the development of disease is unknown [12]. Toxin translocation across the GI tract is not mediated via a receptor dependent process and it may be, for the most part, associated with the direct attachment of the VTEC organism to the mucosal cells, at the site of closest apposition of eukaryotic and prokaryotic membranes. If such a scenario proves to be the case, an insoluble toxin receptor analogue may not have access to toxin prior to translocation into the host. Identification of patients at the hemorrhagic colitis stage and systemic administration of a soluble receptor analogue might well prevent the renal endothelial cell toxin targeting which subsequently may develop into HUS.

4. *Helicobacter pylori*glycolipid recognition and pathogenesis

4.1. Introduction

Helicobacter pylori has gone from being a subject of medical incredulity and ridicule to an acknowledged infection in half the population of the world. Histological localization studies provide the most definitive evidence for a specific receptor for the attachment of this organism to host cells. In gastric biopsies *H. pylori* can be seen associated with the mucus and with the surface of mucosal cells within the antrum but no association with duodenal cells or with duodenal metaplasia within the antrum is seen.

4.2. Adhesins

Several receptor–ligand interactions have been implicated in the mechanism of *H. pylori* host cell attachment and, for the most part, these involve surface carbohydrates of the host cell and in turn, these are for the most part, concerned with glycolipid recognition [58]. The first adhesin/receptor system implicated in *H. pylori* adhesion was the recognition of sialic acid [59]. Such sialyl residues were expressed both on glycoproteins and GM_3 ganglioside. The protein responsible for this sialic acid binding specificity was subsequently cloned [60] but was found to be a lipoprotein within the cell and not a surface exposed adhesin [61]. Interestingly, multimeric 3′ sialyl lactose has been shown to inhibit *H. pylori* binding to several cell lines in vitro and proposed as a possible clinical therapeutic [62]. Sialated polyglycosyl ceramides have also been shown to bind *H. pylori* [63] but this is independent of the putative sialo adhesin mentioned above. The same group has shown binding to a subpopulation of lactosyl ceramide species containing hydroxy sphingosine and hydroxylated fatty acids [64]. Binding of *H. pylori* to the Lewis b blood group structures (expressed on both glycolipids and glycoproteins) has also been reported [65], particularly for organisms of Helicobacter strains termed type 1, in which the vacuolating toxin is expressed. Type 1 *H. pylori* strains are more frequently isolated from symptomatic patients, whereas the type 2 strain are isolated most frequently from patients without gastric symptoms. The adhesin responsible for this Lewis b recognition has recently been identified by a receptor crosslinking procedure [66] and the gene identified from the genome sequence. Since this protein is more frequently expressed in the more pathogenic strains, it provides a new focus for the prevention of clinical disease. In particular, it will be of interest to determine what host cell signal transduction pathways are disturbed following cell surface Le^b binding. The role of Le^b binding in clinical infections is contentious. Although the incidence of ulcers is related to blood group status [67], the colonization with *H. pylori* is not [68]. Several groups have reported the lack of

any role for these oligosaccharides in host cell attachment [69,70], although the subsequent inflammatory response may be related to Lewis status [70]. Thus it is possible that the recognition of Lewis b plays a role in pathogenesis but not necessarily initial eukaryotic cell attachment [71]. However, a transgenic mouse model in which the Le fucosyl transferase is expressed in gastric mucosal cells has been developed [72]. In this model, Le^b plays a crucial role in *H. pylori* mucosal attachment, which in turn is a requirement for the development of gastritis. Only clinical strains expressing the Le^b adhesin were found bound to the mouse gastric mucosal cells after challenge and only such mucosa showed gastritis [73]. Gastric inflammation correlated with the presence of anti Le^x antibodies, suggesting the initiation of an autoimmune reaction by the closely attached organisms, which themselves bear Le^x oligosaccharides within their LPS [74]. However in man, no relationship between *H. pylori* and host Lewis status has been found [75].

H. pylori is a hemagglutinin and several receptor species were proposed based on the ability to agglutinate erythrocytes of different species [76,77]. Our original receptor binding specificities were carried out on the extracts of human erythrocytes and we found that phosphatidyl ethanolamine was the major species within the lipid extract bound [78,79]. No difference between red cells of different blood group status was found. In comparison to PE binding, no recognition of Lewis blood group glycolipids were seen. These binding studies were carried out under microaerophilic conditions but at neutral pH. Gangliotriaosyl ceramide and gangliotetraosyl ceramide, in addition to phosphatidyl ethanolamine, were bound but these species were not detected in red cells or Hep2 cells to which *H. pylori* had been shown to bind. Ganglio series glycolipid are also poorly represented in the lipid extract of the human gastric mucosa [80]. PE expression correlated with the eukaryotic cell binding of *H. pylori* in vitro [81,82]. Although many cells express surface PE, the majority of PE is normally found on the inner leaflet of the plasma membrane bilayer. However, on induction of apoptosis, one of the earliest markers is the loss of the plasma membrane phospholipid asymmetry. This has resulted in the use of annexin 5 which binds to phosphatidyl serine being routinely used as a marker of apoptotic cells. However, in addition to the increased exposure of phosphatidyl serine, exposure of phosphatidyl ethanolamine has been shown to be increased following induction of apoptosis [83] as we had predicted [84]. Interestingly, infection with *H. pylori* has been found to increase the (normally low) percentage of apoptotic cells within the gastric mucosa [85] allowing the speculation that Helicobacter binding to gastric mucosal cells results in the induction of apoptosis in those cells and this then facilitates the binding of more *H. pylori* organisms to the cells.

4.2.1. Assay conditions for receptor binding

H. pylori is a microaerophilic organism. In order to determine the clinically relevant binding specificity of this organism, it appears appropriate to determine the binding specificity under microaerophilic conditions, when the organism remains alive, as opposed to conditions of normal oxygen tension when the organism is dead. Most binding studies thus far reported, however, have not been carried out under such conditions. In addition, the organism's primary niche is highly acidic by nature and therefore, for binding studies to meaningfully reflect the in vivo situation, they must be resistant to conditions of pH 2. This condition has also not been tested in any of the receptor binding studies described above. It should be noted, however, that in the case of Le^b recognition, where such considerations were breached in the most excessive manner [65], that, at least for the transgenic mouse model, binding to Le^b oligosaccharide is the primary determinant for host gastric tissue binding [73].

4.2.2. Stress response induces a change in carbohydrate binding specificity

As mentioned above, *H. pylori* shows many different adhesin–receptor interactions and it is likely that binding to host tissue is a multi-faceted process. However, we were particularly intrigued by reports of *H. pylori* binding to sulfatides by tlc overlay, since under our conditions, we were not able to show sulfatide binding by these organisms. In separate studies, we were investigating the interaction between an hsp70 expressed on the surface of male germ cells and sulfatide [86]. In light of the acidic nature of the site of colonization of this organism, we investi-

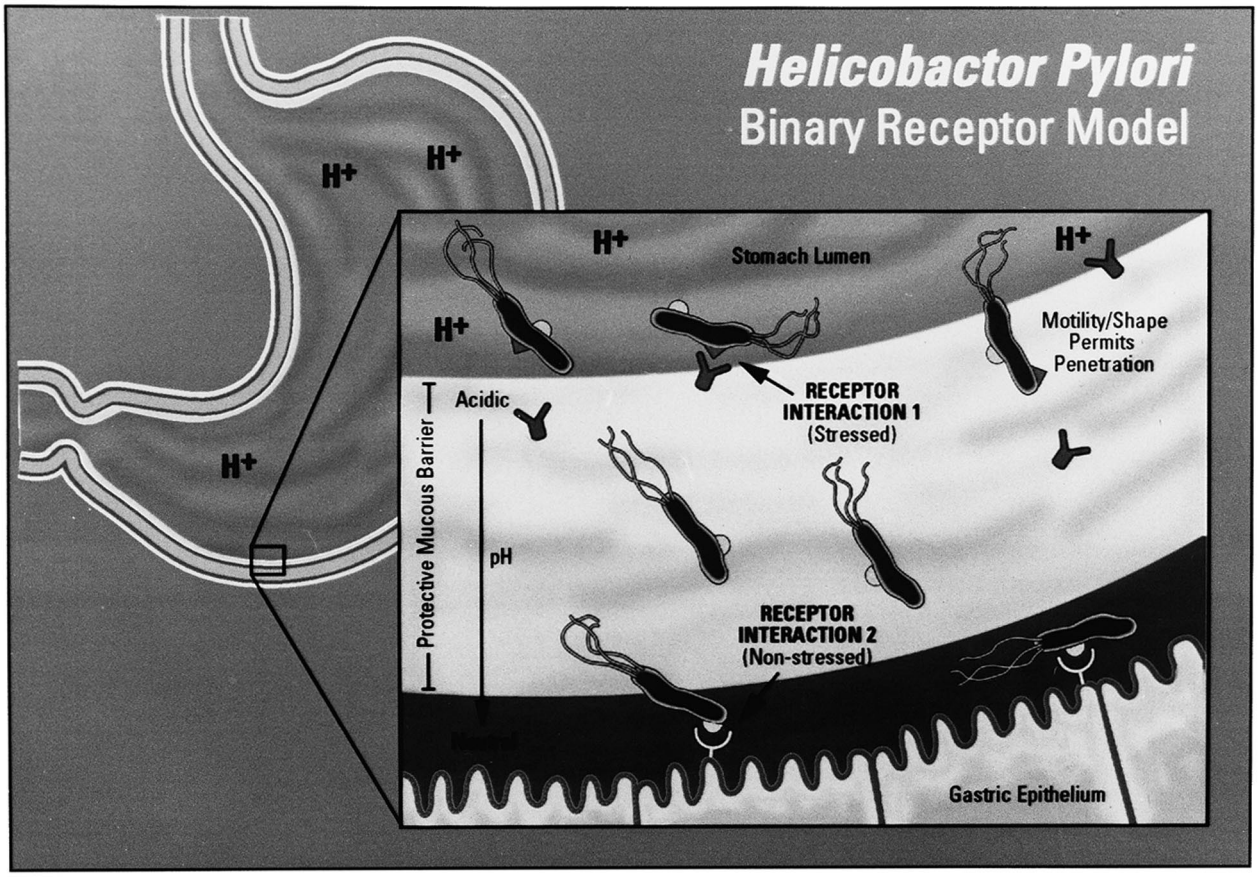

Fig. 2. Binary receptor model for *H. pylori* attachment. Initial antral colonization occurs at acid pH which induces a stress response in *H. pylori* to expose surface hsps to mediate sulfatide binding. Sulfatide (and other sulfate galactolipids) is contained within the mucous layer. Sulfatide binding can thus mediate mucus attachment to prevent removal of *H. pylori* with the stomach contents. Sulfatide, however, is not covalently attached to the mucus, so binding may still allow active penetration of the mucous layer, down a pH gradient to the mucosal surface for attachment to the epithelial cells at approaching neutral pH.

gated whether acid induced a stress response in *H. pylori* to result in the surface expression of an hsp70 which could then mediate sulfatide recognition. This hypothesis turned out to be the case and we were able to show that brief incubation of *H. pylori* at pH 2.5 resulted in an alteration in the glycolipid binding specificity of this organism to include sulfatides, and that this binding specificity could be prevented by inclusion of inhibitors of protein synthesis or by treating the bacteria with anti-hsp70 antibodies [87]. A similar, though not so marked, change in binding specificity to include sulfatide was seen following brief heat shock of the *H. pylori* organisms, again indicating that the sulfatide recognition was the result of a stress response in *H. pylori*. We have subsequently cloned the hsp70 from *H. pylori* and shown it to be preferentially upregulated by pH rather than heat-induced stress [88]. Thus a **binary receptor model** is proposed (Fig. 2) in which initial colonization of the acid stomach resulted in the expression of heat-shock proteins on the cell surface of this organism which bound to sulfated glycolipids contained within the mucous layer. This binding then prevents removal of the organism by the shear forces of gastric peristalsis. Thus the high motility and spiral structure of the organism, together perhaps with the fact that the sulfated glycolipids are not covalently incorporated into the mucus, allows penetration of the mucous layer and access to the gastric epithelium where the pH approaches neutrality, and binding could then occur by an alternative interaction possibly involving PE recognition.

This model is the first example of environmental regulation of carbohydrate specificity and illustrates

the importance of carrying out microbial receptor binding assays under physiological conditions. Our finding that fresh clinical isolates do not bind sulfatide but that after prolonged in vitro culture, sulfatide binding can be detected, suggests that organisms in culture undergo a stress response as a result of suboptimal conditions [88] and raises the important question of the validity of studying strains continuously maintained in culture. In terms of clinical pathology, it is noteworthy that the inhibitory activity of oligosaccharide analogues to prevent *H. pylori* cell attachment varied markedly between fresh and maintained strains, and the strain for which the genome has been sequenced [89] was not representative of the overall response [62].

The expression of cell surface heat shock proteins to mediate sulfatide recognition is a theme that is repeated often in both prokaryotic and eukaryotic cell attachment. Mycoplasma attachment to sulfatide in vitro is inhibited with anti-hsp70 antibodies. Sulfatide binding is induced in *Haemophilus influenzae* following heat shock and this binding is inhibited in the presence of anti-hsp70 [90] and hsp70s are expressed on the surface of *Chlamydia trachomatis* [91]. In eukaryotic systems hsp70 is expressed on the surface of male germ cells to mediate sulfatide binding [92] and an hsp analogue is also expressed on the surface of sea urchin eggs [93] which may serve a similar function.

Since glycolipids are key components in the pathogenesis of both verotoxin and *H. pylori*, the generation of soluble glycolipid derivatives which can mimic the receptor activity of membrane glycolipids is a potential avenue for the generation of new approaches to the prevention of bacterial disease.

Acknowledgements

Studies from my laboratory have been supported by MRC Grants MT 13073 and MT 12559, and NIH Grant R01 DK52098.

References

[1] C.A. Lingwood, Can. J. Biochem. Cell Biol. 63 (1985) 1077.
[2] N. Tanphaichitr, J. Smith, S. Mongkolsirikieart, C. Gradil, C.A. Lingwood, Dev. Biol. 156 (1993) 164.
[3] N. Tanphaichitr, A. Tayabali, C. Gradil, S. Juneja, M.C. Leveille, C.A. Lingwood, Mol. Reprod. Dev. 32 (1992) 17.
[4] A. Thrall, P. Maly, J. Lowe, J. Biol. Chem. 270 (1995) 21437.
[5] Q. Lu, P. Hasty, B. Shur, Dev. Biol. 181 (1997) 257.
[6] K.A. Karlsson, Annu. Rev. Biochem. 58 (1989) 309.
[7] C.A. Lingwood, S. Schramayr, P. Quinn, J. Cell Physiol. 142 (1990) 170.
[8] C.A. Lingwood, in: R. Bell, Y.A. Hannun, A. Merrill Jr. (Eds.), Advances in Lipid Research, Academic Press, San Diego, 1993, p. 189.
[9] P.M. Griffin, in: M.J. Blaser, P.D. Smith, J.I. Ravdin, H.B. Greenberg, R.L. Guerrant (Eds.), Infections of the Gastrointestinal Tract, Raven Press, New York, 1995, p. 739.
[10] P.M. Griffin, S.M. Ostroff, R.V. Tauxe, K.D. Greene, J.G. Wells, J.H. Lewis, P.A. Blake, Ann. Intern. Med. 109 (1988) 705.
[11] P. Rowe, E. Orrbine, G. Wells, P. McLaine, J. Pediatr. 119 (1991) 218.
[12] D.W.K. Acheson, R. Moore, S. De Breucker, L. Lincicome, M. Jacewicz, E. Skutelsky, G.T. Keusch, Infect. Immun. 64 (1996) 3294.
[13] M.A. Karmali, M. Petric, C. Lim, P.C. Fleming, G.S. Arbus, H. Lior, J. Infect. Dis. 151 (1985) 775.
[14] M. Jacewicz, H. Clausen, E. Nudelman, A. Donohue-Rolfe, G.T. Keusch, J. Exp. Med. 163 (1986) 1391.
[15] C.A. Lingwood, H. Law, S. Richardson, M. Petric, J.L. Brunton, S. DeGrandis, M. Karmali, J. Biol. Chem. 262 (1987) 8834.
[16] T. Waddell, S. Head, M. Petric, A. Cohen, C.A. Lingwood, Biochem. Biophys. Res. Commun. 152 (1988) 674.
[17] T. Waddell, A. Cohen, C.A. Lingwood, Proc. Natl. Acad. Sci. USA 87 (1990) 7898.
[18] M.S. Jacewicz, M. Mobassaleh, S.K. Gross, K.A. Balasubramanian, P.F. Daniel, S. Raghavan, R.H. McCluer, G.T. Keusch, J. Infect. Dis. 169 (1994) 538.
[19] L.L.W. Cooling, K.E. Walker, T. Gille, T.A.W. Koerner, Infect. Immun. 66 (1998) 4355.
[20] K. Yoshimura, J. Fujii, T. Yutsuda, R. Kikuchi, S. Shirahata, S. Yoshida, Thromb. Haemost. 80 (1998) 529.
[21] M. Bitzan, S. Richardson, C. Huang, B. Boyd, M.A. Karmali, in: 2nd International Symposium and Workshop on Verocytotoxin (Shiga-like)-producing *Escherichia coli* infections, Bergamo, Italy, 1994, p. 25.
[22] C.A. Lingwood, Nephron 66 (1994) 21.
[23] B. Boyd, G. Tyrrell, M. Maloney, C. Gyles, J. Brunton, C. Lingwood, J. Exp. Med. 177 (1993) 1745.
[24] T. Obrig, C. Louise, C. Lingwood, B. Boyd, L. Barley-Maloney, T. Daniel, J. Biol. Chem. 268 (1993) 15484.
[25] C.B. Louise, T.G. Obrig, Infect. Immun. 60 (1992) 1536.
[26] C.B. Louise, T.G. Obrig, Infect. Immun. 59 (1991) 4173.
[27] N.C.A.J. van der Kar, L.A.H. Monnens, M. Karmali, V.W.M. van Hinsbergh, Blood 80 (1992) 2755.
[28] N.C.J. van der Kar, T. Kooistra, M. Vermeer, W. Lesslauer,

L.A.H. Monnens, V.W.M. van Hinsbergh, Blood 85 (1995) 734.
[29] P. van Setten, L. Monnens, R. Verstraten, L. van der Heuvel, V. van Hinsberg, Blood 88 (1996) 174.
[30] V.L. Tesh, B. Ramegowda, J.E. Samuel, Infect. Immun. 62 (1994) 5085.
[31] B. Ramegowda, V.L. Tesh, Infect. Immun. 64 (1996) 1173.
[32] A. Pellizzari, H. Pang, C.A. Lingwood, Biochemistry 31 (1992) 1363.
[33] B. Boyd, Z. Zhiuyan, G. Magnusson, C.A. Lingwood, Eur. J. Biochem. 223 (1994) 873.
[34] A. Kiarash, B. Boyd, C.A. Lingwood, J. Biol. Chem. 269 (1994) 11138.
[35] S. Arab, C. Lingwood, J. Cell Physiol. 177 (1998) 646.
[36] D. Newburg, P. Chaturvedi, E. Lopez, S. Devoto, A. Feyad, T. Cleary, J. Infect. Dis. 168 (1993) 476.
[37] A.K. Hughes, P.K. Stricklett, D.E. Kohan, Kidney Int. 54 (1998) 426.
[38] M. Turner, K. Kher, T. Rakussan, L. D'Angelo, S. Kapur, D. Selby, P. Ray, Pediatr. Nephrol. 11 (1997) 161.
[39] P. Dickie, J.E.M. Felser, J. Bryant, J. Silver, N. Marinos, A. Notkins, Virology 185 (1991) 109.
[40] X.-H. Liu, C.A. Lingwood, P. Ray, Kidney Int. 55 (1999) 554.
[41] N. Strömberg, Arch. Oral Biol. 35 (1990) 131S.
[42] E. Bock, M.E. Breimer, A. Brignole, G.C. Hansson, K.-A. Karlsson, G. Larson, H. Leffler, B.E. Samuelsson, N. Strömberg, C. Svanborg Edén, J. Biol. Chem. 260 (1985) 8545.
[43] S. Haataja, K. Tikkanen, J. Liukkonen, C. François-Gerard, J. Finne, J. Biol. Chem. 268 (1993) 4311.
[44] K. Bock, T. Freijd, J. Kihlberg, G. Magnusson, Carbohydr. Res. 176 (1988) 253.
[45] S. Haataja, K. Tikkanen, U. Nilsson, G. Magnusson, K. Karlsson, J. Finne, J. Biol. Chem. 269 (1994) 27466.
[46] P.G. Nyholm, G. Magnusson, Z. Zheng, R. Norel, B. Binnington-Boyd, C.A. Lingwood, Chem. Biol. 3 (1996) 263.
[47] P.M. St. Hilaire, M.K. Boyd, E.J. Toone, Biochemistry 33 (1994) 14452.
[48] S. Head, M. Karmali, C.A. Lingwood, J. Biol. Chem. 266 (1991) 3617.
[49] G. Nyberg, N. Strömberg, A. Jonsson, K.-A. Karlsson, S. Normark, Infect. Immun. 58 (1990) 2555.
[50] P.-G. Nyholm, J.L. Brunton, C.A. Lingwood, Int. J. Biol. Macromol. 17 (1995) 199.
[51] P.-G. Nyholm, I. Pascher, Biochemistry 32 (1993) 1225.
[52] H. Ling, A. Boodhoo, B. Hazes, M. Cummings, G. Armstrong, J. Brunton, R. Read, Biochemistry 37 (1998) 1777.
[53] H. Shimizu, R.A. Field, S.W. Homans, A. Donohue-Rolfe, Biochemistry 37 (1998) 11078.
[54] W.W. Picking, J.A. McCann, A. Nutikka, C.A. Lingwood, Biochemistry 38 (1999) 7177.
[55] M. Mylvaganam, C.A. Lingwood, J. Biol. Chem. 274 (1999) 20725.
[56] M. Mylvaganam, C. Lingwood, Biochem. Biophys. Res. Commun. 257 (1999) 391.
[57] G.D. Armstrong, P.N. McLaine, P.C. Rowe, in: J.B. Kaper, A.D. O'Brien (Eds.), *Escherichia coli* O157:H7 and Other Shiga Toxin-producing *E. coli* Strains, American Society for Microbiology, Washington, DC, 1998, p. 374.
[58] C.A. Lingwood, Curr. Biol. 2 (1998) 695.
[59] D.G. Evans, D.J. Evans, J.J. Moulds, D.Y. Graham, Infect. Immun. 56 (1988) 2896.
[60] D. Evans, T. Karjalainen, D. Evans, D. Graham, C.-H. Lee, J. Bacteriol. 175 (1993) 674.
[61] P.W. O'Toole, L. Janzon, P. Doig, J. Huang, M. Kostrzynska, T.J. Trust, J. Bacteriol. 177 (1995) 6049.
[62] P.M. Simon, P.L. Goode, A. Mobasseri, D. Zopf, Infect. Immun. 65 (1997) 750.
[63] H. Miller-Podraza, M.A. Milh, J. Bergström, K.-A. Karlsson, Glycoconj. J. 13 (1996) 453.
[64] J. Angstrom, S. Teneberg, M.A. Milh, T. Larsson, I. Leonardsson, B.-M. Olsson, M.O. Halvarsson, D. Danielsson, I. Naslund, Å. Ljungh, T. Wadstrom, K.-A. Karlsson, Glycobiology 8 (1998) 297.
[65] T. Borén, P. Falk, K.A. Roth, G. Larson, S. Normark, Science 262 (1993) 1892.
[66] D. Ilver, A. Arnqvist, J. Ogren, I.-M. Frick, D. Kersulyte, E.T. Incecik, A. Covacci, L. Engstrand, T. Borén, Science 279 (1998) 373.
[67] J.H. Kurata, B.M. Haile, Clin. Gastroenterol. 13 (1984) 289.
[68] A. Mentis, C.C. Blackwell, D.M. Weir, C. Spiliadis, A. Dailianas, N. Skandalis, Epidemiol. Infect. 106 (1991) 221.
[69] M. Clyne, B. Drumm, Gastroenterology 113 (1997) 72.
[70] M.A. Heneghan, A.P. Moran, K.M. Feeley, E.L. Egan, J. Goulding, C.E. Connolly, C.F. McCarthy, FEMS Microbiol. Lett. 20 (1998) 257.
[71] B. Su, P.M. Hellstrom, C. Rubio, J. Celik, M. Granstrom, S. Normark, J. Infect. Dis. 178 (1998) 1379.
[72] P.-G. Falk, L. Bry, J. Holgersson, J. Gordon, Proc. Natl. Acad. Sci. USA 92 (1995) 1515.
[73] J.L. Guruge, P.G. Falk, R.G. Lorenz, M. Dans, H.-P. Wirth, M.J. Blaser, D.E. Berg, J.I. Gordon, Proc. Natl. Acad. Sci. USA 95 (1998) 3925.
[74] B.J. Appelmelk, I. Simoons-Smit, R. Negrini, A.P. Moran, G.O. Aspinall, J.G. Forte, T. DeVries, H. Quan, T. Verboom, J.J. Maaskant, P. Ghiara, E.J. Kuipers, E. Bloemena, T.M. Tadema, R.R. Townsend, K. Tyagarajan, J.M. Crothers Jr., M.A. Monteiro, A. Savio, J. De Graaff, Infect. Immun. 64 (1996) 2031.
[75] D.E. Taylor, D.A. Rasko, R. Sherburne, C. Ho, L.D. Jewell, Gastroenterology 115 (1998) 1113.
[76] T. Wadström, J.L. Guruge, S. Wei, P. Alejung, A. Ljungh, in: P. Malfertheiner, H. Ditschuneit (Eds.), *Helicobacter pylori*, Gastritis and Peptic Ulcer, Springer, Berlin, 1990, p. 96.
[77] J.A. Armstrong, N. Cooper, C.S. Goodwin, J. Robinson, S.H. Wee, M. Burton, V. Burke, J. Med. Microbiol. 34 (1991) 181.
[78] C.A. Lingwood, H. Law, A. Pellizzari, P. Sherman, B. Drumm, Lancet ii (1989) 238.
[79] C.A. Lingwood, M. Huesca, A. Kuksis, Infect. Immun. 60 (1992) 2470.

[80] H. Natomi, T. Saitoh, K. Sugano, M. Iwamori, M. Fukayama, Y. Nagai, Lipids 28 (1993) 737.
[81] M. Dytoc, L. Fedorko, M. Huesca, B. Gold, M. Louie, S. Crowe, C. Lingwood, J. Brunton, P. Sherman, Infect. Immun. 61 (1993) 448.
[82] B.D. Gold, M. Dytoc, M. Huesca, D. Philpott, A. Kuksis, S. Czinn, C.A. Lingwood, P.M. Sherman, Gastroenterology 109 (1995) 692.
[83] K. Emoto, N. Toyama-Sorimachi, H. Karasuyama, K. Inoue, M. Umeda, Exp. Cell Res. 232 (1997) 430.
[84] J. Busse, E. Hartmann, C.A. Lingwood, J. Infect. Dis. 175 (1996) 77.
[85] N.L. Jones, P.T. Shannon, E. Cutz, H. Yeger, P.M. Sherman, Am. J. Pathol. 151 (1997) 1695.
[86] D. Mamelak, C. Lingwood, Glycoconj. J. 14 (1997) 715.
[87] M. Huesca, S. Borgia, P. Hoffman, C.A. Lingwood, Infect. Immun. 64 (1996) 2643.
[88] M. Huesca, A. Goodwin, A. Bhagwansingh, P. Hoffman, C.A. Lingwood, Infect. Immun. 66 (1998) 4061.
[89] J.-F. Tomb, O. White, A.R. Kerlavage, R.A. Clayton, G.G. Sutton, R.D. Fleischmann, K.A. Ketchum, H.P. Klenk, S. Gill, B.A. Dougherty, K. Nelson, J. Quackenbush, L. Zhou, E.F. Kirkness, S. Peterson, B. Loftus, D. Richardson, R. Dodson, H.G. Khalak, A. Glodek, K. McKenney, L.M. Fitzegerald, N. Lee, M.D. Adams, E.K. Hickey, D.E. Berg, J.D. Gocayne, T.R. Utterback, J.D. Peterson, J.M. Kelley, M.D. Cotton, J.M. Weidman, C. Fujii, C. Bowman, L. Watthey, E. Wallin, W.S. Hayes, M. Borodovsky, P.D. Karp, H.O. Smith, C.M. Fraser, J.C. Venter, Nature 388 (1997) 539.
[90] E. Hartmann, C.A. Lingwood, Infect. Immun. 65 (1997) 1729.
[91] J.E. Raulston, C.H. Davis, D.H. Schmiel, M.W. Morgan, P.B. Wyrick, J. Biol. Chem. 268 (1993) 23139.
[92] J. Boulanger, D. Faulds, E.M. Eddy, C.A. Lingwood, J. Cell. Physiol. 165 (1995) 7.
[93] K.R. Foltz, J.S. Partin, W.J. Lennarz, Science 259 (1993) 1421.