Corynebacterium diphtheriae surface proteins as adhesins to human erythrocytes

Andrêa Vieira Colombo a, Raphael Hirata Jr. a, Cláudio Marcos Rocha de Souza a, Luiz Henrique Monteiro-Leal b, José Oswaldo Previato c, Luiz Carlos Duarte Formiga c, Arnaldo Feitosa Braga Andrade a, Ana Luíza Mattos-Guaraldi a,*

a Disciplina de Microbiologia e Imunologia, Faculdade de Ciências Médicas, Universidade do Estado do Rio de Janeiro, Av. 28 de Setembro, 87 Fundos, 3 andar, Vila Isabel, CEP 20 551-030 Rio de Janeiro, Brazil
b Instituto de Biologia, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, Brazil
c Instituto de Microbiologia Prof. Paulo de戈es, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil

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Abstract

Corynebacterium diphtheriae strains express non-fimbrial surface proteins able to recognize and bind to specific host cells receptors. Protein extracts were obtained from bacterial cells by mechanical process and ammonium sulfate precipitation at 25 and 45% (w/v) saturation. SDS-PAGE analysis of the extracts detected two polypeptide bands of 67 and 72 kDa, named 67-72 p. The 67-72 p, rabbit anti-67-72 p IgG antibodies as well as human gastric mucin, N-acetylneuraminic acid and N-acetyl D-glucosamine molecules were able to inhibit bacterial hemagglutination. Hemagglutination assays using 67-72 p-coated latex beads and Western blot analysis of biotin-labeled 67-72 p and erythrocyte receptors demonstrated the binding of 67-72 p to human erythrocyte membranes. Immunolabeled colloidal gold-A protein transmission electron microscopy using anti-67-72 p revealed a diffuse distribution of non-fimbrial 67-72 p on the surface of C. diphtheriae strains of both sucrose-fermenting and non-fermenting biotypes. Non-fimbrial lectin-like surface 67-72 p may play a role as adhesins in bacterial attachment thereby facilitating the early steps in pathogenesis of both toxigenic and non-toxigenic C. diphtheriae. ß 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Microbiological Societies.

Keywords: Diphtheria; Non-fimbrial lectin-like adhesin; Surface-associated hemagglutinin; Corynebacterium diphtheriae

1. Introduction

Immunization with diphtheria toxoid protects against toxigenicity but not against colonization and invasion by Corynebacterium diphtheriae. Differences in diphtheria mortality can be in part due to differences in the nature of the microorganisms. The degree of adhesion proved to be an important factor of pathogenicity for both toxigenic and non-toxigenic strains [1,2]. Main primary approaches and new developments in the study of the molecular basis of the adhesion process were recently reviewed along with a discussion of the potential importance of hemagglutinins, exposed sugar residues, hydrophobins and trans-sialidase enzyme as adhesins of the sucrose-fermenting and non-fermenting biotypes [2]. Nevertheless, the molecular basis of the interactions involved in the process and its importance in virulence remained unresolved.

The adhesion of C. diphtheriae isolated from patients with different forms of infection was previously observed in experimental models using human erythrocytes [1,3] and buccal epithelial cells [4]. Differences in the degree of hemagglutination and adhesion to glass suggested a diversity in the expression of C. diphtheriae adhesins [5,6]. Recent studies demonstrated the expression of receptor molecules specific for lectins with an affinity for sialic acid, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, galactose and mannose-like sugar residues on surface of C. diphtheriae strains [7].

Fimbral structures were observed on the surface of one
C. diphtheriae strain and were also associated with bacterial hemagglutination [8]. However, studies with purified C. diphtheriae fimbral proteins or antisera to such fimbral preparations were not performed in order to directly implicate these structures, as mediators of bacterial attachment to host surfaces. Earlier investigations suggested the binding of host carbohydrates to bacterial non-fimbrial proteins [6]. The aim of the present study was to assess the participation of non-fimbrial surface proteins in the interaction of C. diphtheriae with human erythrocyte membranes.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Toxigenic C. diphtheriae var. mitis were used: two sucrose-fermenting strains isolated from throat (strain 241) and blood (strain HC01) of patients in Rio de Janeiro, Brazil; one non-fermenting strain (CDC-E8392) from Centers for Disease Control and Prevention, Atlanta, GA, USA. Strains were grown in trypticase soy broth (TSB; Difco Laboratories, Detroit, MI, USA) for 48 h at 37°C and stored in GC-medium with 10% glycerol at −20°C [9,10].

2.2. Bacterial cell surface proteins, antiserum and IgG antibodies

Bacterial surface proteins were isolated from the CDC-E8392 strain by a mechanical process and ammonium sulfate precipitation at 25 and 45% saturation (w/v) [8,11]. Antisera were prepared in adult male rabbits as previously described [12]. Rabbit IgG antibodies were purified by 33% (w/v) ammonium sulfate precipitation and by high performance liquid chromatography (HPLC; Pharmacia LKB Biotechnology, Uppsala, Sweden) [13]. Amount and purity of the proteins were estimated by a modified Lowry method [14] and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; Bio-Rad, Richmond, CA, USA), respectively [15].

2.3. Coating of carboxylated polystyrene beads with proteins

Carboxylated microspheres (0.8–1.0 μm in diameter; Sigma Chemical Co., St. Louis, MO, USA) were coated with bacterial proteins (67-72 p) or bovine serum albumin (BSA) as previously described [16].

2.4. Biotin-labeled protein binding to erythrocyte membrane

Purified 67-72 p and BSA were labeled with biotin (Sigma) as described elsewhere [12]. Human erythrocyte suspensions (1% v/v) were prepared in 0.02 M phosphate-buffered saline (PBS, pH 7.2) and incubated with 1 μg ml⁻¹ biotin-labeled 67-72p or BSA at 37°C for 1 h. Erythrocyte membrane fractions obtained by lysis with distilled water were submitted to SDS-PAGE and transferred to a nitrocellulose membrane for 90 min at 100 V. Protein blots blocked with 5% skim milk solution were incubated with streptavidin-peroxidase conjugate (Sigma) and developed with 0.3% (v/v) hydrogen peroxide, 1 mg ml⁻¹ 3,3’ diaminobenzidine and 1 mg ml⁻¹ imidazole [12].

2.5. Hemagglutination and inhibition assays

Bacterial protein solution (0.75 mg ml⁻¹) and microspheres coated with either 67-72 p or BSA (3.5 × 10⁹ beads ml⁻¹) were used in hemagglutination assays. Bacterial hemagglutination with untreated or neuraminidase-treated human erythrocytes (0.5%) was also performed. Erythrocyte suspensions (5% v/v) in PBS, pH 6.0, were incubated with 0.2 U ml⁻¹ Clostridium perfringens neuraminidase (type X; Sigma) at 37°C for 30 min. [3,7].

Bacterial 67-72 p (0.75 mg ml⁻¹), anti-67-72 p IgG (1 mg ml⁻¹) and BSA (0.75 mg ml⁻¹) were investigated for their ability to inhibit bacterial hemagglutination. Hemagglutination inhibition assays were also performed with thyroglobulin and human gastric mucin glycoproteins and the saccharide hapten, α-mannose, α-galactose, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, α-fucose and N-acetyleneuraminic acid (all from Sigma) [17].

2.6. Transmission electron microscopy (TEM)

Bacterial cells and 67-72 p preparations were negatively stained with 1% potassium phosphotungstate prior to examination in a Zeiss EM 906 transmission electron microscope [8]. For immunogold electron microscopy (IEM) studies, bacterial cells were incubated with rabbit anti-67-72 p IgG (2 mg ml⁻¹) at 37°C for 30 min, treated with 5–10 μm colloidal gold–protein A (Sigma) at 37°C for 30 min. and negatively stained as described above. Control experiments were performed by either omitting or replacing rabbit anti-67-72 p IgG by non-immune rabbit sera [18].

3. Results

3.1. Characterization of bacterial surface proteins by SDS-PAGE

Two main polypeptide chains with molecular masses of 67 and 72 kDa (67-72 p) were detected by SDS-PAGE in bacterial extracts (Fig. 1). Samples not treated with β-mercaptoethanol revealed bands similar in size to bands presented by reduced 67-72 p. Extraction procedures allowed approximately 8 mg of protein extract from 60 g (wet weight) of bacterial cells.
3.2. Adhesion activity of the 67 and 72 kDa polypeptides to human erythrocyte membranes

Western blot analysis of erythrocyte membrane proteins previously exposed to biotinylated bacterial surface proteins revealed two bands of 67 and 72 kDa, indicating binding activity of both bacterial surface polypeptides to erythrocyte receptors (Fig. 1, lane c).

3.3. Adhesion of 67-72 k-coated beads to human erythrocytes

Latex microspheres conjugated with 67-72 k were able to agglutinate human erythrocytes (titer 8). Latex microspheres conjugated to BSA did not react with erythrocytes. The interaction of 67-72 k or BSA-coated beads with erythrocyte surfaces was visualized by light microscopy (Fig. 2).

3.4. Inhibition of hemagglutinating activity

As shown in Table 1, bacterial surface 67-72 k obtained from non-fermenting C. diphtheriae CDC-E8392 strain and anti-67-72 k IgG inhibited the interaction of the homologous strain and heterologous sucrose-fermenting HC01 strain with human erythrocytes at minimal concentration of 0.36 μg ml⁻¹ and 0.48 μg ml⁻¹, respectively. Similar concentrations of BSA did not inhibit bacterial hemagglutination. Concentrations > 750 μg ml⁻¹ of 67-72 k suspension were unable to cause direct agglutination of human erythrocytes.

Human gastric mucin and thyroglobulin inhibited the hemagglutination of both strains tested (Table 2). The non-fermenting CDC-E8392 strain presented higher affinity to both macromolecules. The hemagglutinating activity of the sucrose-fermenting HC01 strain was also sensitive to N-acetylneuraminic acid (15.4 mg ml⁻¹) and N-acetyl-D-glucosamine (6.25 mg ml⁻¹) residues.

The treatment of erythrocytes with C. perfringens neuraminidase enhanced the hemagglutination of 241 strain but did not influence the hemagglutination of HC01 and CDC-E8392 strains (Table 3).

3.5. Visualization of 67-72 k adhesins on bacterial surface

The surface 67-72 k labeled with colloidal gold particles was observed by TEM (Fig. 3). The 67-72 k or BSA-coated beads with erythrocyte surfaces was visualized by light microscopy (Fig. 2).

Table 1

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Minimum inhibitory concentration (μg ml⁻¹)</th>
</tr>
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<tr>
<td></td>
<td>BSA 67-72 k Anti-67-72 k IgG</td>
</tr>
<tr>
<td>非蔗糖发酵的</td>
<td></td>
</tr>
<tr>
<td>CDC-E8392</td>
<td>&gt; 750 0.36 0.48</td>
</tr>
<tr>
<td>蔗糖发酵的</td>
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<tr>
<td>HC01</td>
<td>&gt; 750 0.36 0.48</td>
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*aAverage of three experiments that differed by ≤ 10%.

Table 2

<table>
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<th>Inhibitor</th>
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<tr>
<td></td>
<td>HCO1 strain CDC-E8392 strain</td>
</tr>
<tr>
<td>Human gastric mucin</td>
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</tr>
<tr>
<td>Thryglobulin</td>
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</tr>
<tr>
<td>N-acetylneuraminic acid</td>
<td>15.40 &gt; 30.90</td>
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<tr>
<td>N-acetyl-D-glucosamine</td>
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<tr>
<td>N-acetyl-L-galactosamine</td>
<td>&gt; 22.10 &gt; 22.10</td>
</tr>
<tr>
<td>D-galactose</td>
<td>&gt; 18.00 &gt; 18.00</td>
</tr>
<tr>
<td>D-mannose</td>
<td>&gt; 18.00 &gt; 18.00</td>
</tr>
<tr>
<td>L-fucose</td>
<td>&gt; 16.40 &gt; 16.40</td>
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</tbody>
</table>

*aAverage of three experiments that differed by ≤ 10%.
Table 3

Influence of neuraminidase treatment of human erythrocytes on the hemagglutinating activity of *C. diphtheriae* strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Titer of erythrocyte agglutination on</th>
<th>Untreated</th>
<th>NA/Nase-treated</th>
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<tr>
<td>241</td>
<td></td>
<td>1</td>
<td>32</td>
</tr>
<tr>
<td>HC01</td>
<td></td>
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<td>8</td>
</tr>
<tr>
<td>CDC-E8392</td>
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<td>32</td>
<td>32</td>
</tr>
</tbody>
</table>

*Average of three experiments that differed by ≤10%.*

*C. perfringens* neuraminidase (type X; Sigma).

4. Discussion

The multifactorial nature of *C. diphtheriae* adhesion was considered once bacterial strains adhered to several substrates in varied intensities [2]. Fimbriae of several bacterial species including *Corynebacterium* spp. were described as <30 kDa protein structures sensitive to heating at 100°C, detergent and trypsin treatment [8,11,19]. *C. diphtheriae* hemagglutinins were found to be resistant to the above treatments [1,6] suggesting the involvement of non-fimbrial molecules in the hemagglutinating activity of the species. Non-fimbrial adhesins expressed on bacterial cell surfaces of several species may play a role in attachment to host cells [19].

Present data demonstrated that bacterial surface polypeptide chains >30 kDa (67-72 p) could promote the interaction of sucrose-fermenting and non-fermenting *C. diphtheriae* strains with human erythrocyte membranes. The hemagglutinating activity of 67-72 p adsorbed to latex beads was visualized by light microscopy. Results of Western blot assays confirmed the binding activity of both 67 and 72 kDa polypeptides to erythrocyte receptors. The hemagglutinating activity of proteins that behave as a homopolymer was previously observed with other bacterial species [16]. Further studies are necessary to investigate whether the 67-72 p corresponds to one single or two distinct protein molecules.

The presence of non-fimbrial 67-72 p adhesins on *C. diphtheriae* cell surface as observed by IEM, was independent of the sucrose fermentation biotype and the hemagglutinating properties of the strains. IEM assays did not reveal the presence of fimbrial structures on the surface of hemagglutinating *C. diphtheriae* var. mitis strains. The lack of fimbriae was also observed in previous studies with negatively stained preparations of European hemagglutinating *C. diphtheriae* var. gravis strains [1]. The fact that no fimbriae were observed does not exclude their presence on other isolates or under other experimental conditions.

Hemagglutinating activity was not detected in supernatants of *C. diphtheriae* cultures indicating that hemagglutinins are not released from bacterial surfaces, in contrast to other respiratory tract pathogens [20]. The adhesion process in *C. diphtheriae* seem to involve structures exposed at the bacterial surface (colonization and adhesion factors) that are able to recognize and bind to specific host cells receptors.

Many biologically important host–pathogen interactions appear to involve binding of host carbohydrates to bacterial lectin-like proteins [21]. The inhibition of the hemagglutinating activity of *C. diphtheriae* by the highly glycosylated macromolecules tested (thyroglobulin and human gastric mucin) suggested a lectin-like activity by 67-72 p surface-associated adhesins.

The results of the hemagglutination inhibition tests by the monosaccharides suggested the participation of *N*-acytelneuraminic acid and *N*-acetyl-D-glucosamine residues in the interaction of glycoconjugates exposed on human cell membranes with the invasive (HC01) *C. diphtheriae* strain isolated from blood of a patient with fatal endocarditis [22]. However, *N*-acytelneuraminic acid and *N*-acetyl-D-glucosamine residues did not inhibit the hemagglutination of the CDC-E8392 strain (from which 67-72 p was extracted). These monosaccharides were probably incapable to dislodge the carbohydrate competitor ligand site exposed on the erythrocyte surface.

Previous investigations demonstrated that *C. diphtheriae* cell surface contained sialic acid residues at the terminus. Sialic acid terminal constituents were mainly expressed on the surface of the sucrose-fermenting biotype (241) strain [7,23]. The neuraminidase treatment of erythrocytes led to increased hemagglutination by the 241 *C. diphtheriae* strain, possibly through the reduction of the electrostatic repulsion and/or by exposition of other sugar residues, including subterminal galactose units, that may be recognized by bacterial lectins as previously described with other species [21,24]. However, desialylation of erythrocyte
membranes did not influence the hemagglutinating activity mediated by the 67-72 p on CDC-E8392 and HC01 strains. The precise characterization of the cell receptors specific for the bacterial 67-72 p hemagglutinins is still necessary.

The selective recognition of human erythrocytes by bacterial lectin-like proteins may facilitate C. diphtheriae colonization and dissemination of infection. Experiments using animal models could lead to further confirming data that C. diphtheriae interacts directly with erythrocytes in vivo. The understanding of the factors determining the molecular basis of the colonization process will improve our potential to control infections caused by both toxigenic and non-toxigenic C. diphtheriae strains. Future investigations are necessary to determine the definite role of the 67-72 p surface-associated hemagglutinins as colonization binding factor of C. diphtheriae.

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