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The Erythrocyte and Epithelial Cell Receptors for *Haemophilus influenzae* Are Expressed Independently

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The Anton blood group antigen has been shown to be the erythrocyte receptor for *Haemophilus influenzae*. Cord erythrocytes, which lack the Anton antigen, were not agglutinated by *H. influenzae* (L. van Alphen, J. Poole, and M. Overbecke, FEMS Microbiol. Lett. 37:69–71, 1986). Twenty-eight erythrocyte suspensions from newborns less than 4 days old were also not agglutinated, but 23 of 56 erythrocyte suspensions from 4- to 50-day-old newborns and 23 of 35 erythrocyte suspensions from older infants were agglutinated. Positive hemagglutination correlated with the presence of the Anton antigen on the erythrocytes for 163 of 173 ($P < 0.0001$). Adherence of *H. influenzae* to buccal epithelial cells obtained from six newborns within 3 days after birth was as strong as that found with adult epithelial cells, whereas the erythrocytes from five of six of these newborns were not agglutinated by the bacteria. Adherence of *H. influenzae* to epithelial cells of 15 donors was not inhibited by anti-Anton serum. Moreover, *H. influenzae* carrying fimbriae adhered to epithelial cells of an Anton-negative donor. From these results we conclude that the age at which the erythrocyte receptor for *H. influenzae* is expressed is the same as for the Anton antigen, but that the receptor on the epithelial cells is already expressed at birth and is not identical to the Anton antigen.

Haemophilus influenzae is an important cause of respiratory tract infections (bronchitis, otitis, nasopharyngitis, sinusitis, and pneumonia). The encapsulated type b strains are also commonly isolated from cerebrospinal fluid and blood of patients with meningitis, epiglottitis, and cellulitis (11). The infection is presumed to be preceded by nasopharyngeal colonization (5). Bacteria which adhere to nasopharyngeal epithelial cells carry fimbriae. Adherence is generally accepted as the first step in the establishment of the bacteria on the mucous membranes (1). Fimbriation is general among *H. influenzae* (7). Fimbriated *H. influenzae* bacteria adhere to human epithelial cells and bind to human erythrocytes, resulting in hemagglutination (HA) (7).

We have shown previously that the Anton blood group antigen is involved in HA by *H. influenzae* isolated from cerebrospinal fluid, sputum, and throat (12). Cord erythrocytes are not agglutinated by *H. influenzae*, and neither do they express the Anton antigen (12). Adults with Anton-negative erythrocytes are rare (8).

Interestingly, newborns are rarely infected by *H. influenzae* (11). Fothergill and Wright have already shown that the majority of these very young infants acquire bactericidal antibodies against *H. influenzae* b from their mothers transplacentally (2), which antibodies are important for the protection of these children in the first months of life. We analyzed whether these infants may also be protected by the lack of a receptor for *H. influenzae*. Therefore we determined the age at which the erythrocytes of children become agglutinatinable by fimbriated *H. influenzae* and express the Anton antigen. In addition, the age dependence of adherence of *H. influenzae* to pharyngeal epithelial cells was compared with that of the Anton antigen on erythrocytes in these infants.

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MATERIALS AND METHODS

Strains and growth conditions. *H. influenzae* b strain 770235 was isolated from cerebrospinal fluid and stored at −70°C in peptone containing 15% glycerol (13, 14). At that time it did not express fimbriae as observed with electron microscopy after negative staining (strain 770235f$^-$). It was cultured overnight on chocolate agar plates in a humid atmosphere containing 5% CO_2 at 37°C. Strongly hemagglutinating bacteria (strain 770235f$^+$) were selected by enrichment on human erythrocytes as described before (5, 12). The bacteria had hundreds of fimbriae per cell as observed with electron microscopy. A dilution of 1:256 (HA titer) of 10^{10} bacteria per ml in 15 mM sodium phosphate–0.1 M NaCl, pH 7.4 (PBS), still agglutinated a 3% (vol/vol) erythrocyte suspension (12). The number of bacteria per milliliter was determined from the viable count of appropriate dilutions.

Blood samples and epithelial cells. Blood samples were obtained from 119 individual newborns and infants in a neonatal unit and a pediatric department of the Academic Medical Centre, Amsterdam, The Netherlands. Blood was collected in tubes containing EDTA.

Pharyngeal epithelial cells from newborns were obtained by scraping the posterior of the oral cavity with a cotton stick. Mainly pharyngeal epithelial cells were obtained. The cells were washed three times with PBS and suspended in Eagle-Hanks medium (GIBCO Laboratories) at a density of 10^5 cells per ml. Pharyngeal epithelial cells from 15 healthy adults with Anton-positive and Anton-negative erythrocytes were used as controls.

HA assay. Three microliters of blood were diluted in 50 μl of PBS in a round-bottom tube. A bacterial suspension (50 μl of 10^{10} bacteria per ml) was added, and HA was performed by a standard tube blood grouping assay. The results were read with a dark-field microscope as described before (12). Adult and cord erythrocytes were used as positive and

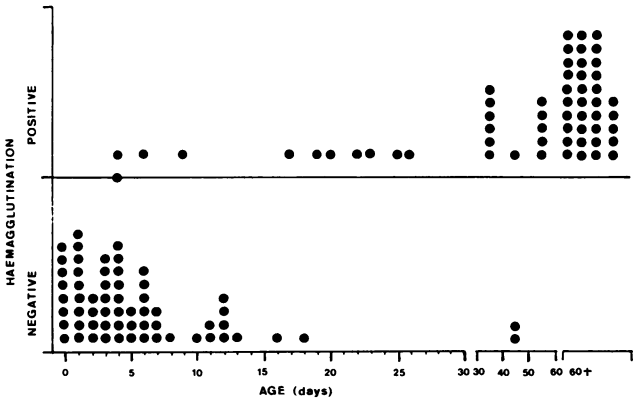

FIG. 1. HA of erythrocytes of newborns and infants by *H. influenzae* 770235f$^+$. Each dot represents an individual (n = 119).

negative controls, respectively. HA by strain 770235f$^-$ was always negative. The blood samples were analyzed under code.

Blood grouping. Anton typing of erythrocytes was performed by the indirect antiglobulin technique with anti-human immunoglobulin G (8).

Adherence of bacteria to epithelial cells. Bacteria of *H. influenzae* 770235f$^+$ were suspended in Eagle-Hanks medium to a density of 10^{10} cells per ml. One hundred microliters of the epithelial cell suspension was mixed with 10 μl of bacterial suspension and incubated for 1 h at 37°C. The epithelial cells with adherent bacteria were recovered from the incubation mixture by centrifugation for 2 min at 500 × g, washed with PBS, and centrifuged for 2 min at 500 × g. This procedure was repeated twice to remove unattached bacteria. After the cells were suspended in 50 μl of PBS, preparations were made on glass slides. After drying, fixation of cells was performed in methanol for 5 min. The bacteria were stained with the immunoperoxidase staining technique of Terpstra et al. (10). Murine ascites with monoclonal antibodies from hybridoma cell line 5HA5 were used as the first antibody at a dilution of 1:1,000. These antibodies are specific for outer membrane protein a (molecular weight 47,000) of *H. influenzae*. The production and description of these monoclonal antibodies will be published elsewhere. Anti-mouse immunoglobulin G–horseradish peroxidase conjugate (Sigma Chemical Co.) was used as the second antibody. The epithelial cells were counterstained with a 0.1% solution of methylene blue in water. The number of bacteria on 20 cells was counted under the microscope to calculate the mean number per cell.

Inhibition of adherence was performed by preincubating the epithelial cells with undiluted anti-Anton serum for 2 h at 37°C before the cells were incubated with bacteria. These sera did not agglutinate erythrocytes or bacteria (12).

Statistical methods. The correlation between HA by *H. influenzae* and the occurrence of the Anton antigen was analyzed with the chi-square test. Random selection of single blood samples from individuals from whom multiple isolates were obtained was done by the stoichiastic distribution method.

RESULTS

Dependence of HA by *H. influenzae* on the age of the erythrocyte donor. A preliminary survey on 10 blood samples confirmed that cord blood erythrocytes are not agglutinated by *H. influenzae* and showed that the erythrocytes from 3-month-old infants were positive. A total of 119 samples were collected from individual children, 96 of whom were younger than 3 months. The results of the HA experiments are summarized in Fig. 1, which shows that the 35 samples from individuals above 2 months were all positive. The 28 samples from newborns less than 4 days were all negative. The relative number of positives in the age group 5 days to 2 months increased gradually between days 4 and 50 after birth. We could not find a relationship between the appearance of HA and length of gravidity. Weak HA was exceptional, indicating either that the test is very sensitive or that the expression of the receptor for *H. influenzae* is an all-or-nothing effect.

Correlation between HA and the expression of the Anton antigen. A total of 176 blood samples were analyzed independently by one of us (J.P.) for the presence of the Anton antigen. The correlation between HA and the expression of the Anton antigen is shown in Table 1. This series included 77 HA-positive blood samples, 95 negative samples, and 4 intermediate samples. A good correlation was found for 163 of 176 samples (93%). Seven were inconclusive (4%), and six did not correlate (3%). This correlation was highly significant (χ^2 = 127; 1 df; $P < 0.0001$), indicating that the Anton antigen on erythrocytes is expressed concomitantly with the expression of the receptor for *H. influenzae* on erythrocytes.

Age-related adherence of *H. influenzae* to epithelial cells. Bacteria of *H. influenzae* 770235f$^+$ adhered strongly to pharyngeal epithelial cells of 15 Anton-positive adults. More than 100 bacteria were found per epithelial cell from 9 of 15, 50 to 100 bacteria were found per epithelial cell from 5 of 15, and 10 to 50 bacteria were found per epithelial cell from 1 of 15 donors under the experimental conditions used. Bacteria without fimbriae (strain 770235f$^-$) hardly adhered (fewer than four bacteria per cell). Examples of both are shown in Fig. 2A and B, respectively. In each preparation a minority of the epithelial cells did not bind bacteria, and a considerable variation from cell to cell was observed. These results are in agreement with data from other investigators (3, 6, 9).

We compared the HA of erythrocytes by *H. influenzae* 770235f$^+$ with adherence to pharyngeal epithelial cells to determine whether both receptors are expressed at the same age. Pharyngeal epithelial cells and erythrocytes from six newborns, collected on the same day, were examined. HA was negative for five out of six blood samples, whereas adherence to epithelial cells from all six newborns was comparable to that seen with well-adhering cells of adults (>500 bacteria per cell; Fig. 2C).

Inhibition experiments and relationship of adherence to erythrocyte Anton type. Epithelial cells of 15 Anton-positive adults were incubated with anti-Anton serum, which was shown to inhibit HA when erythrocytes were incubated in the same way (12). Adherence to epithelial cells was not influenced by anti-Anton serum, since the epithelial cells of 9 of 15 donors bound more than 100 bacteria of strain 770235f$^+$ per cell, cells of 5 of 15 donors bound 50 to 100

TABLE 1. Correlation between the expression of the receptor for *H. influenzae* (HA) and the Anton antigen on erythrocytes

HA expression	No. with Anton antigen expression			
	+	±	−	*n*
+	73	2	2	77
±	1		3	4
−	4	1	90	95

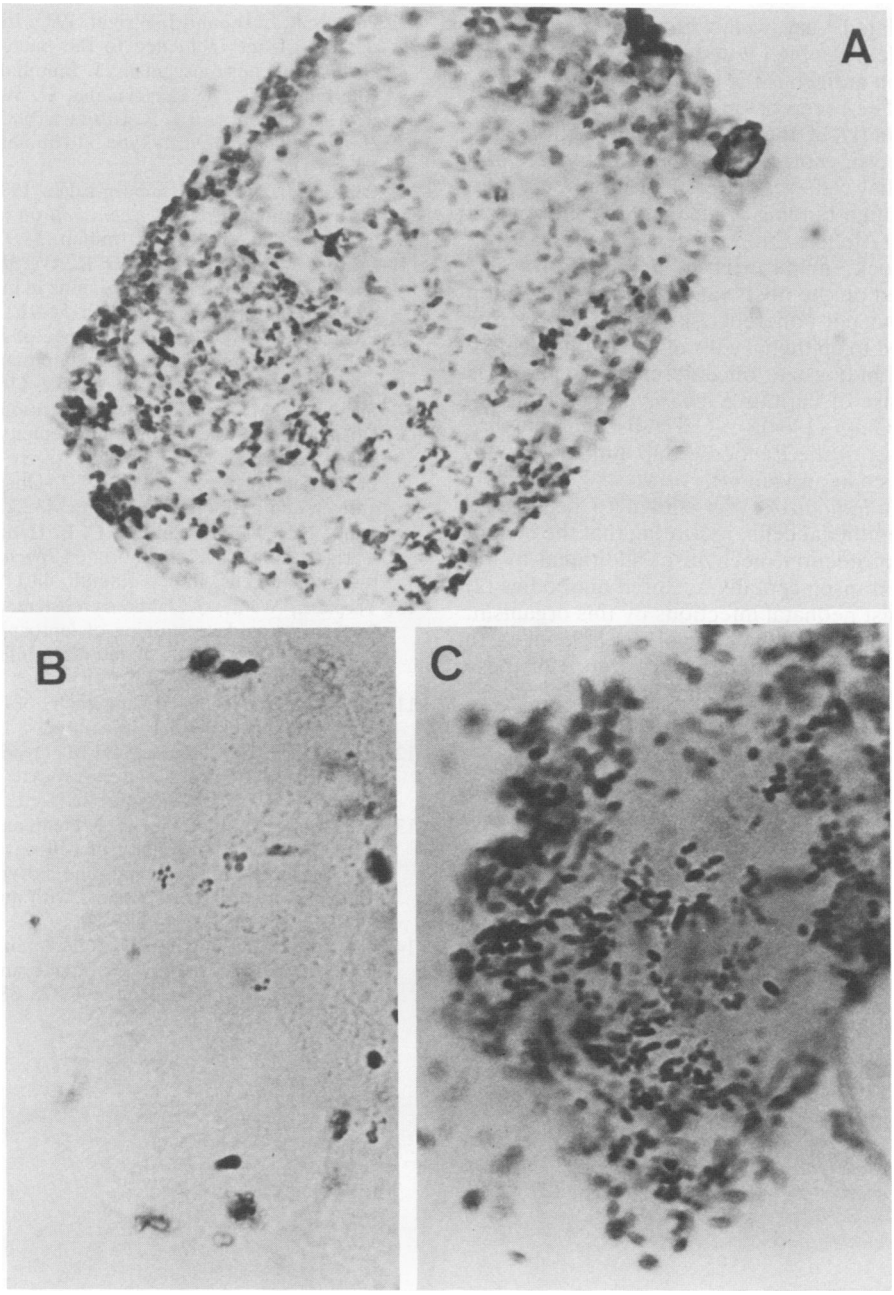

FIG. 2. Adherence of *H. influenzae* to oropharyngeal epithelial cells from an adult incubated with *H. influenzae* 770235f$^+$ (A) or 770235f$^-$ (B). (C) Epithelial cells from a newborn incubated with strain 770235f$^+$.

bacteria, and cells of one donor bound 10 to 50 bacteria. Strain 770235f$^-$ still did not bind under these conditions. Adherence was also tested with epithelial cells obtained from an Anton-negative donor. These cells appeared as positive in adherence as epithelial cells from an Anton positive donor for the strain carrying fimbriae. Bacteria without fimbriae were negative, indicating that fimbriae are also important for the adherence of bacteria to epithelial cells of an Anton-negative donor.

DISCUSSION

Binding of bacteria to human erythrocytes resulting in HA is easily detected and has been used to analyze the host cell components involved in the interaction between the bacteria and their receptors. We have previously shown that the Anton blood group antigen is the erythrocyte receptor for *H. influenzae* b and nontypable strains from cerebrospinal fluid and sputum (12). This antigen is not expressed on cord erythrocytes.

We determined the age at which the erythrocyte receptor becomes expressed and whether the presence of this receptor and the Anton antigen correlates with adherence of the bacteria to oropharyngeal epithelial cells. The erythrocyte receptor was expressed early in life, varying between days 3 and 50 (Fig. 1). This variation in appearance was not related to the duration of the gravidity. Expression of the erythro-

cyte receptor correlated very well with expression of the Anton antigen (Table 1), which extends our previous conclusion that the Anton antigen is the erythrocyte receptor for *H. influenzae* (12). The receptor on the epithelial cells was expressed independently of the erythrocyte receptor, suggesting that the receptor molecules for binding by *H. influenzae* on the epithelial cells are different from the erythrocyte receptors, although binding is mediated by fimbriae in both cases (L. van Alphen, N. van den Berghe, and L. Geelen-van den Broek, manuscript in preparation). This conclusion was based on the observation that (i) anti-Anton sera inhibited HA but not adherence and (ii) *H. influenzae* with fimbriae adhered to epithelial cells of an Anton-negative donor. A common binding site on different molecules has been found in the case of various *Escherichia coli* isolates, for which the α-D-Gal-*p*-(1→4)β-D-Gal-*p*-disaccharide has been shown to be part of the P blood group antigens P^k, P_1, and P_2 and the Forssman antigen (4).

In conclusion, *H. influenzae* was shown to be able to adhere to newborn epithelial cells, indicating that the lack of adherence is not a protection mechanism additional to the humoral defense by transplacentally acquired antibodies (2) in the newborn against neonatal infections by this organism. The results also showed that binding of *H. influenzae* to erythrocytes is not a perfect model for adherence of these bacteria to the receptor on the epithelial cells.

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