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RECURRENT RESPIRATORY INFECTIONS IN DOWN SYNDROME AND CONTROL SUBJECTS: A PROSPECTIVE STUDY OF BACTERIAL ADHESION

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ABSTRACT

Background*:* bacterial adhesion is the first crucial step in bio film formation, which often serves as a source for recurrent infections. Since many individuals with Down syndrome have recurrent respiratory tract infections, we studied the bacterial adhesion in a group of them in comparison to control subjects.

Study design: prospective, controlled study.

Methods: we stratified all subjects into four groups for the presence/absence of recurrent respiratory infections. Exfoliated buccal epithelial cells were incubated with *Staphylococcus aureus ATCC 12598* and the mean number of adhering bacteria per cell was counted under scanning electron microscope.

Results*:* the Down syndrome and the control groups prone to recurrent respiratory infections showed both higher adhesion values in comparison to groups without recurrent respiratory tract infections. Moreover, the Down subjects without recurrent respiratory infections had bacterial adhesion values to buccal epithelial cells similar to control subjects.

Conclusions*:* The increased bacterial adhesion values in Down and control subjects prone to recurrent respiratory tract infections is probably attributable to an increased expression of cell receptors for bacteria.

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Keywords: Bacterial adhesion; Staphylococcus aureus; buccal cells; Down syndrome; recurrent respiratory tract infections.

BACKGROUND

Down syndrome (DS) (OMIM # 190685) is one of the most common genetic causes of intellectual disability in children. In Italy, the prevalence is approximately 1 in 2.000 live born infants.

DS in characterized by a variety of dysmorphic features associated with several immunological impairments that might explain the increased incidence of leukemia, celiac disease, hypothyroidism and diabetes mellitus.

In DS children, the occurrence of recurrent respiratory tract infections ($RRTI^+$) is a common problem encountered in daily clinical practice, interesting up to 77 % of patients aged 5-16 years [1].

People with DS have a higher mortality rate for respiratory infections, approximately twice than that observed in individuals of the same age [2]. The respiratory infections and congenital heart defects (CHD) are the two most frequently reported causes of death [3-5]: in particular, bronchopneumonia is the most frequent cause in older subjects, while for younger ones is heart disease [6].

In their course of life, this population also has a higher incidence of respiratory infections, which tends to increase with age [3]. The upper respiratory tract is the most affected: rhinosinusitis, pharyngitis and effusive otitis media are common.

The lower respiratory tract infections are one of the most common causes of hospitalization in this population, regardless of the coexistence or not of CHD [7, 8] and respiratory infections, especially pneumonia, are more frequent when the subjects are institutionalized [9].

Even through some DS children may not present frequent infections ($RRTI^-$), the course of their infective illnesses might be prolonged and have increased severity compared with non-DS children [10, 11]. Compared to $RRTI^-$, $RRTI^+$ children show decreased mental and motor development, impaired hearing, more behavioural problems and lower scores on most health-related quality of life [12].

Published guidelines [13] include recommendations for the follow-up of the increased susceptibility to respiratory infections in DS, especially in children.

A few data on microbial etiology are available in patients with DS, although it is considered that most respiratory infections, especially of the upper respiratory tract, are caused by viruses, as it is for general population. Respiratory viral infections, on the other hand, predispose the individual to bacterial secondary infections by promoting bacterial adhesion [14, 15]. *Streptococcus pneumoniae*, *Haemophilus influentiae* and *Moraxella catharralis* are the three most common bacteria known to cause otitis media and pneumonia in children [16].

The pathophysiology underlying the increased risk for respiratory disease in DS remains unclear, even if multiple abnormalities, such as hypotonia, developmental delay, craniofacial shape and CHD may contribute [17]. In addition, several studies have shown some abnormalities in the immunoglobulin levels, as decreased salivary IgA and IgG and IgG4 subclass deficiency is common finding in DS patients with recurrent infections [18, 19]. Chronic aspiration is another cause that could explain the recurrent respiratory symptoms in DS patients [20].

Our Departments are reference centres for the VIVI DOWN Association, ONLUS of Milan, Italy, and we carry out regular monitoring visits to DS patients aged between 5 and 65 years: despite the above mentioned epidemiological reports, in our experience we observed that only a part of them undergoes recurrent respiratory tract infections, while others have an incidence of respiratory infections quite similar to subjects without Down syndrome.

The aim of the study was to investigate bacterial adhesion in individuals with DS compared to a group of healthy control subjects, stratified for the presence of RRTI. This is a new approach in the understanding of the respiratory infections in DS, studying the bacterial adhesion to epithelial cells, according to the mostly spread model that involves the use of exfoliated buccal epithelial cells incubated with a bacterial strain, for example, *Staphylococcus aureus* as bacterium representative of Gram+ infections. Similarly, the bacterial adhesion test of *Streptococcus pneumoniae* has previously demonstrated to have a good positive predictive value for acute otitis media in prone children [21].

In the sequence of steps that occur in an infectious process, bacterial adhesion to mucosal surfaces is the first essential event before the colonization: bacteria adhere to host tissues through a mechanism of specific interaction "adhesin-receptor" which leads to the formation of a stable relationship between bacterium and cell. Bacterial adhesins are lectins located on pili, flagelli, fimbriae and outer membrane proteins targeting highly specific carbohydrates within the epithelial extracellular matrix [22]. Expression of the microbial receptors on host epithelium is, therefore, key to the establishment of a respiratory infection. The expression of receptors on host cells is a dynamic process, according to the functional state of the cell. We speculated that one possible cause of recurrent respiratory infections may be the expression on the surface of oropharyngeal epithelial cells of a greater number of receptors for bacterial adhesins.

PATIENTS AND METHODS

One hundred voluntaries were recruited and stratified as follows: 30 DS patients $RRTI^+$ (group A), 30 DS patients $RRTI^-$ (group B), 15 subjects without DS $RRTI^+$ (group C) and 25 healthy subjects $RRTI^-$ (group D).

$RRTI^+$ were defined as more than 6 respiratory infections in one year or more of three episodes of upper respiratory infection per month between September and April or three or more lower respiratory infections per year [23].

The DS subjects were recruited among those belonging to the VIVI DOWN Association that relate to our hospital for a periodical survey; subjects without DS, identified as controls, were mainly medical specialists or nurses working in our departments or patients performing a pulmonary visit as check-up.

Accurate medical history data have been collected, focused on the incidence of infectious episodes of upper and lower respiratory tract and it was assessed the objectivity ENT.

All subjects were evaluated in a phase of clinical stability, should not have taken antibiotics in the three weeks prior and were not to have acute infections.

The protocol was approved by the Ethic Committee of Ospedale Maggiore of Milan, Italy (4/3/2010, n. 505).

For each subject, a written consensus to participation was obtained signed by themselves or their parents when minors.

Collection of epithelial cells

The experimental model, credited in the literature to study bacterial adhesion of the respiratory tract, involves the use of exfoliated buccal epithelial cells: they are collected with a sterile plastic spatula according to the method of Ellen and Gibbons [24]; after rinsing the oral cavity with sterile water, the mucosa of each cheek was gently scraped three to five times. Epithelial cells were dislodged with 5 ml phosphate buffered saline (PBS) and passed through a needle (0.3 mm in diameter) to disrupt cellular aggregates; then they were washed three times to free them from debris and non-adherent bacteria by low-speed centrifugation.

PBS was added to the washed epithelial cells suspension to give 10^5 cells/ml, as determined by direct microscopic counts (interference contrast microscopy) in a Bürker chamber.

Cell suspensions were stored at -20 °C until use.

Bacterial strain and culture condition

We utilized cell suspension of *Staphylococcus aureus* ATCC 12598 as bacterium representative of Gram+ infections. We prepared cell suspension of *Staphylococcus aureus* from overnight cultures in trypticase soy broth at 37°C. The organisms were harvested, washed three times in PBS by centrifugation at 500 g and then adjusted to 1 x 10^8/ ml. The number of bacteria per millilitre was estimated by interference contrast microscopy in a Petroff-Hausser chamber.

In vitro assay*:* cells-bacteria interaction

The adherence of bacteria to epithelial cells was investigated by mixing 1:1 volumes of standardized suspensions of bacteria and epithelial cells in polystyrene tubes. The tubes were rotated end-over-end at 10 rev. /min for 45 min at 37°C.

Each test was performed in duplicate.

Epithelial cells suspensions were also incubated with buffer, instead of bacterial suspension, to provide data on the number of bacteria that will already be attached (natural acquisition) when they are collected.

The epithelial cells were separated from non-adhering bacteria by differential centrifugation at 320 x g for 5 min. according to the method of Fletcher (25).

Scanning electron microscopy

The final epithelial cell pellet was re-suspended in a small quantity of PBS, placed on a round microscope coverslide and allowed to dry. The round coverslide with the cells was then fixed in 2.5 % glutaraldehyde in 0.1 M cacodhylate buffer, pH 7.25 for 30 min. After dehydration in a growing series of alcohols, the coverslides underwent critical point drying and coated with 200 Angstrom units of gold and were counted in a scanning electron microscope (Jeol JSM 6060, Japan).

Data collection and statistical evaluation

The adhesiveness of bacteria to epithelial cells was determined by counting the mean number of adhering bacteria /100 epithelial cells (26). The mean number of adhering bacteria per cell for each experiment was obtained by subtracting the background adhesion (the number of bacteria present on cells incubated with buffer only).

To determine the statistical significance of the different adherence values for *S. aureus* to buccal epithelial cells in the four groups, we employed a univariate general linear model with the belonging group as fixed effect factor, age as covariant, gender as random effect factor and Bonferroni correction for multiple comparisons.

RESULTS

Table 1 shows the characteristics of the four groups; all enrolled subjects had normal objectivity ENT.

Table 1: Characteristics of groups enrolled in the study. Data are expressed as mean ± standard deviation.

Group	Number	Males	Females	Mean age ± SD	Range
A	30	14	16	20.83 ± 11.03	7 – 45
B	30	18	12	28.87 ± 10.83	10 – 53
C	15	3	2	19.40 ± 13.41	8 – 34
D	25	5	20	34.04 ± 14.67	5 – 58

The test strain of *Staphylococcus aureus* ATCC 12598 demonstrated to adhere well to oropharyngeal cells.

The univariate general linear model showed significant differences among the four groups (F=9.43, p=0.046), while gender and age had no effects. The post hoc tests indicated that:

- the healthy controls $RRTI^-$ had adhesiveness values significantly lower than control subjects $RRTI^+$ (group C vs D, p=0.034).
- the DS group $RRTI^-$ showed adhesiveness values lower than DS subjects $RRTI^+$ (group B vs A, p<0.001).
- the DS group $RRTI^-$ showed the same adhesiveness values of the controls $RRTI^-$ (group B vs D, p=NS).
- the DS group $RRTI^+$ showed the same adhesiveness values of the controls $RRTI^+$ (group A vs C, p=NS).

These results were reported in Figure 1.

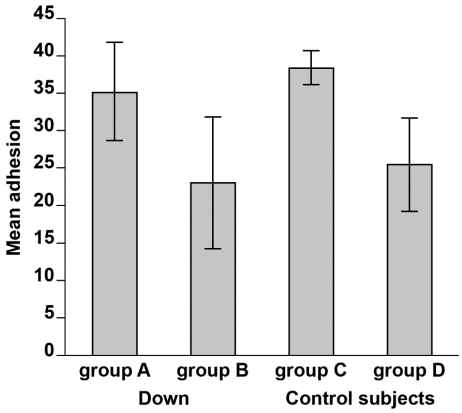

Figure 1: Mean adherence of bacteria to buccal cells ± standard deviation from the four groups of subjects.

Figure 2 shows an example of adhesion to buccal cells obtained from the four groups at scanning electron microscopy.

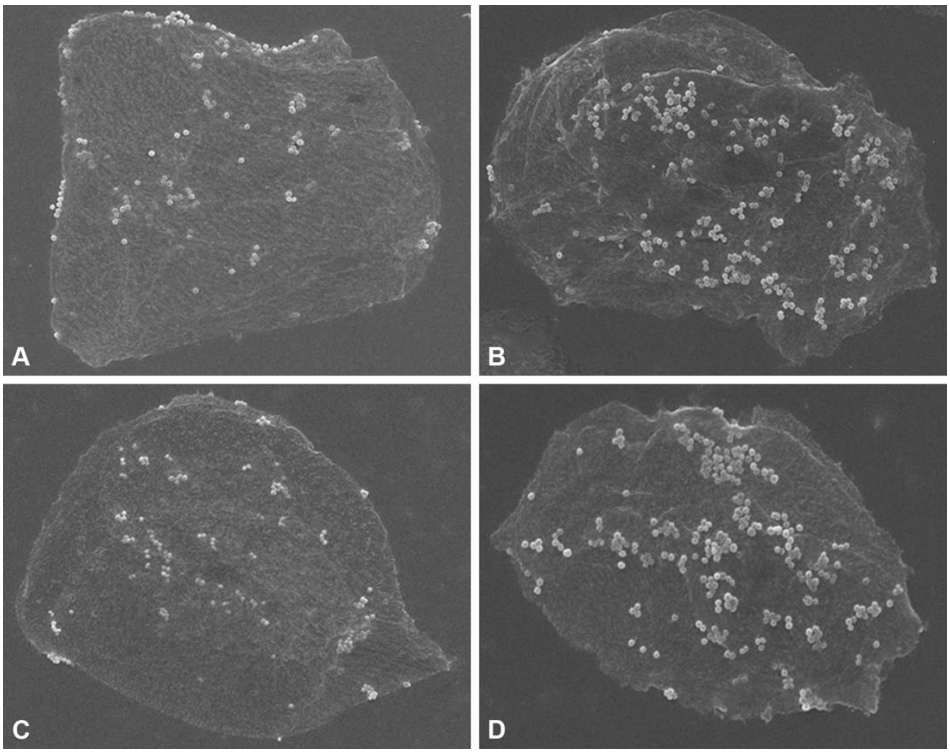

Figure 2 - Example of bacterial adhesion of *Staphylococcus aureus* to buccal cells obtained from healthy subjects without Down syndrome (A), subjects without Down syndrome prone to recurrent respiratory infections (B), Down syndrome without recurrent respiratory infections (C) and Down syndrome prone to recurrent respiratory infections (D).

DISCUSSION

In the general population, the problem of frequent RRTI usually affects pre-school children [27]; in adult subjects this is not frequent, except when some comorbidities exist [28]. Individuals with DS rather represent a particular group of subjects, in which the problem of RRI is particularly relevant during childhood, but it can arise even in adulthood, at least for some of them. Respiratory tract infections in DS are a common problem encountered in daily clinical practice and are still the most important cause of mortality at all ages [29,30].

Cardiovascular and pulmonary diseases account for ~ 75 % of the mortality observed in persons with DS and respiratory disease constitutes a large proportion of the morbidity in DS and contributes to reduced life expectancy [31].

There are several reasons that may explain the high incidence of respiratory infections in the DS subjects, for example: immuno-hematological abnormalities, lymphopenia and eosinopenia [32], function abnormalities of neutrophils [33], low levels of immunoglobulins [18, 34], craniofacial abnormalities affecting predominantly oral

breathing [35], deficit of mucociliary clearance [36] and alterations of the oxidative metabolism determined by high levels of superoxide dismutase [37, 38].

Anatomic abnormalities of the respiratory tree as pulmonary hypoplasia and alterations of the airways [39, 40], as the altered pharyngeal motility which promotes tracheal aspiration [41], are all factors that may explain the susceptibility to respiratory infections in DS.

More often, it can be observed a high frequency of infectious episodes in the upper respiratory tract as rhinitis, sinusitis, middle ear infections, tonsillitis, pharyngitis; sometimes, also in the lower airways as bronchitis and pneumonia.

However, some DS subjects have the same incidence of infectious events than non-DS individuals.

Several investigators have demonstrated that the bacterial binding capacity of epithelial cells of specific tissue sites from infection-prone persons is greater than that of similar cells from persons not prone-infections [42]. For example, the nasal epithelial cells obtained from staphylococcal carriers were shown to bind significantly greater number of *Staphylococcus aureus* than nasal epithelial cells from noncarriers [43, 44]. Oropharynx of hospitalized patients is frequently colonized by Gram-negative bacteria and epithelial cells of colonized patients bind bacteria more avidly than epithelial cells of noncolonized do [45]. Many of clinical conditions that are associated with colonization have been shown to be capable of altering the respiratory mucosal surface increasing its ability to bind bacteria [46].

The first step in bacterial colonization of the respiratory tract involves the binding of a bacterial adhesions to its specific receptor presented on the surface of the host cells. Expression of the microbial receptors on host epithelium is, therefore, key to the establishment of a respiratory infection [47].

We studied the phenomenon of bacterial adhesion in DS in comparison to control groups, stratifying the subjects for the presence/absence of RRTI: this is a new way to deepen the knowledge of RRTI. Our findings demonstrate that DS subjects $RRTI^+$ exhibit higher values of bacterial adhesion to buccal epithelial cells, comparing to DS subjects $RRTI^-$ and non-DS subjects; DS subjects $RRTI^+$ show values of bacterial adhesion similar to non-DS subjects $RRTI^+$. Interestingly, the groups $RRTI^-$, show similar values of bacterial adhesion, demonstrating that DS is not *per se* a prerequisite for increased bacterial adhesion.

One explanation for the highest number of adherent bacteria in DS and non-DS subjects $RRTI^+$ could be the increased expression of cell surface receptors capable of binding to bacteria specifically, although this is an indirect evidence. It is known that bacteria receptors are expressed on the cell surface in a dynamic way, according to the functional state of the cell. It is possible that the same infection in cells induce a state of increased receptor expression by creating the condition for the occurrence of the infectious event. The infection by one pathogen can increase the number of receptors available for another pathogen, as reported for influenza A viral infection that results in heightened expression of receptors for streptococci [48, 49]. Rhinovirus infection has also been shown to significantly increase fibronectin, platetelet activating factor receptor [47] and other molecules promoting adhesion of *Staphylococcus aureus, Streptococcus pneumoniae and Haemophilus influentiae* [50].

We used the *Staphylococcus aureus* adhesion as model of respiratory infections from Gram+, although there is no evidence of a greater number of respiratory infections by this bacterial type in people with DS. A more

complete study could examine the adhesion of other bacterial strains known as respiratory pathogens: in fact, since different bacteria bind different sites on host cells, the bacterial adhesiveness could be different for other bacterial species. Another limit of the present study is the lacking of a direct proof of the number of receptors that would require more complex molecular biochemistry studies.

However, these preliminary results can be of clinical interest in view of screening and monitoring over time subjects at risk for developing recurrent infections.

CONCLUSIONS

Subjects with DS frequently have an increased tendency to respiratory tract infections: the adhesion of bacteria to mucosal surfaces, mediated by the expression of adhesins, is the first event before the nasopharyngeal colonization, the biofilm formation and the following spreading of infection.

Adhesion of *Staphylococcus aureus* to buccal epithelial cells is widely used as a bacterial adherence parameter to study individuals prone to recurrent infections of upper and lower respiratory tract: this is a useful test to follow up and medically support these subjects to prevent deterioration of their situation.

The ability to prevent bacterial adhesion represents an ideal goal to interfere with bacterial pathogenesis and colonization at an early stage and it is also an intriguing topic for future research and development of alternative strategies to antibiotics such as the therapeutic use of receptor analogs targets of bacterial adhesins.

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SUMMARY

- People with DS have a high incidence of respiratory infections in their course of life, which tends to increase with age. Some of them suffer of recurrent respiratory infections, even in adulthood.
- We studied the phenomenon of bacterial adhesion to buccal epithelial cells in DS individuals in comparison to control subjects, stratified for the prevalence/absence of recurrent respiratory infections.
- We observed a higher number of adherent bacteria in DS and non-DS subjects prone to recurrent respiratory infections, but similar bacterial adhesion values in both groups without recurrent respiratory infections.

- Therefore, Down syndrome is not a prerequisite per se for increased bacterial adhesion.
- The increased number of adherent bacteria in subjects prone to recurrent respiratory infections is probably attributable to the increased expression of cell surface receptors capable of binding to bacteria specifically.

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