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# Naturally occurring immune response against bacteria commonly involved in upper respiratory tract infections: analysis of the antigen-specific salivary IgA levels

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**Abstract**

Lyophilized bacterial lysates, which actively stimulate the immune response, are widely used as vaccines or 'biological response modifiers' in subjects with recurrent bacterial respiratory infections. Since vaccines are indicated in the absence or in the presence of a weak constitutive immune response activity, a better knowledge on the 'naturally' occurring antibacterial immune response at the oropharingeal level should be helpful. A study was, therefore, designed to quantify the presence of salivary IgA directed against surface antigens bacteria frequently involved in the pathogenesis of upper respiratory tract infections: *Klebsiella pneumoniae* (KP), *Staphylococcus aureus* (SA), *Streptococcus pyogenes* (SPy), *Morraxella catarrhalis* (MC), *Haemophylus influenzae* (HI), and *Streptococcus pnumoniae* (SPn). In 34 volunteers (21 adults and 13 children), salivary fluid was collected and the presence of microorganism-specific IgA antibodies evaluated by a novel enzyme immuno-assay. In the whole population only 29 and 24% of subjects had IgA directed, respectively, to KP and SA, while the immune-response against other microbes was detectable in a small population ranging from 12 to 15% of all subjects studied. We found higher proportions of individuals with strain specific salivary IgA in the adult than in the pediatric population for all the microorganism evaluated. In addition, in children, the only strain inducing a significant production of specific IgA at oropharingeal level was KP. Interestingly, only ten out of 21 adults and two out 13 children have at least one significantly high antibody titer against one of the bacteria evaluated. Nevertheless, when a group of healthy donors was treated with a polyvalent mechanical bacterial lysate (Ismigen t.), the large majority developed a specific immune-response in the salivary fluid. These results are thus consistent with the good features of the novel enzyme-immunoassay and with a poor frequency of naturally induced specific anti-microbe antibodies in children and in adults despite the presence on recurrent respiratory infections in their clinical history.


*Keywords:* Infection; Immunoglobulin; Bacteria

## 1. Introduction

One of the difficult-to-deal problems in medical practice is the management of the patients with persistent or recurrent upper respiratory tract infections with or without associated chronic bronchial involvement [1]. Since a significant proportions of these subjects do not have detectable underlying etiologic factors explaining the recurrence of symptoms, this condition is frequently explained as a reflection of a familial or individual susceptibility or as the result of repeated exposure to infectious agents, at home or at school [1] for children and at home or at work for adults [2].

The observation that recurrent respiratory infections may be caused by or associated with secretory IgA deficiencies [3], has suggested as 'prophylactic approach' the stimulation of the 'mucosal' immune response against microorganisms commonly involved in the

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pathogenesis of this type of disorders [4]. This working hypothesis has renovated the interest of scientists into a group of apparently obsolete 'biological response modifiers', the bacterial lysates [4]. These drugs have been used in the past in order to improve the efficiency of the immune response both in pediatric and adult patients [5–9]. However, despite the large number of clinical trials indicating that a significant protection against respiratory infection recurrence could be obtained, few data are available on the immunological basis of this protection [10–12]. A non specific enhancement of 'regional' total IgA has been associated to good clinical results [13] but no data is available on the specificity of the immune response. Along this line, the capability to increase total IgA concentrations in salivary fluid may only reflect a 'non-specific' immunomodulatory activity of the bacterial lysates [4]. On the contrary, the demonstration of modifications in the levels of IgA subsets specifically directed against the surface molecules of the microorganisms present in the bacterial lysate could allow to certify that the drug has a specific immunostimulating activity [4,13]. Moreover, this specificity should be strictly related to the possibility to act as immunoprophylactic vaccines. Now, it is common notion that a vaccine is indicated in the absence of a natural immune response, and only in few cases, the presence of a weak constitutive activity has been associated with the need of any further stimulation [14,15].

Thus, a better knowledge on the antibacterial 'naturally' occurring immune response at the oropharingeal level in both children and adults is mandatory in order to foresee a clinical trial using bacterial lysates as immunostimulating and/or immunoprophylactic drugs.

With this background, we designed a study to quantify in a group of children and adults, the natural immune response against bacteria generally involved in the pathogenesis of upper respiratory tract infections, using an original approach to evaluate the presence of salivary IgA directed against surface structures of these microorganisms.

## 2. Material and methods

### 2.1. Subjects

Two populations were studied: 21 volunteers (12 males and ten females, median age 41, ranging from 21 to 65 years), represented the adult study population, while the children population included 13 individuals (nine males and four females, mean age nine, ranging from 7 to 10 years). The narrow range of ages for the pediatric patients was chosen on the basis of the well known maturation of the locoregional immune-response during development. Participating subjects were routinely followed for recurrent respiratory infections but were in stable clinical conditions when evaluated. They did not report upper or lower respiratory infection in the 2 months preceding the study and did not have treatments with any drug (such as corticosteroids) known to interfere with immunoglobulin production in the 4 months preceding the study. Both the patients and the parents or guardians of all children were informed on the aims of the study and gave informed consent. The protocol was approved by the Ethics Committee of each Institution. Finally, a group of ten normal volunteers was treated with a polyvalent bacterial mechanical lysate (Ismigen® tablets, Zambon Italia) to induce the secretion of anti-bacteria IgA at salivary level.

### 2.2. Collection of salivary fluids

The salivary fluid was collected from the individuals belonging to the two groups using a special device (Salivette, Sarstedt, Numbrecht, Germany), according to the producer's instructions. Following collection of the fluid, the saliva saturated polyester swabs were returned to the tube, than centrifuged at $1000 \times g$ for 2 min. The collected fluids, free of cells and food debris, were stored frozen until used to measure the salivary IgA levels specific to bacterial antigens.

### 2.3. Anti-IgA antisera

Horseradish peroxidase-labeled, affinity-purified antisera to human IgA, were obtained from Jackson Laboratories (Listarfish, Milan, Italy). The proper working dilution was defined using a standard block titration technique.

### 2.4. Antigens

The particulate antigens were obtained from the following bacteria strains: *Staphylococcus aureus* (SA), *Streptococcus pyogenes* (SPy), *Klebsiella pneumoniae* (KP), *Haemophilus influenzae* (HI), *Moraxella catarrhalis* (MC), *Streptococcus pnumoniae* (Spn). The purity of the bulk culture was checked using clinical microbiology routine identification procedures. Then, after in vitro expansion of each strain, bacteria were extensively washed then subjected to a mechanical fragmentation using a high pressure valve. Following another washing, the powder was lyophilized and stored at 4 °C. Microscopic examination of fragmented bacteria after Gram staining documented the presence of bacterial debris maintaining the original Gram characteristics. Finally, the antigenicity of these preparations was checked using rocket electrophoresis against a panel of policlonal rabbit anti-SA, anti Spy, anti-KP, anti-HI, anti-MC and anti-Spn. For this aim, the particulate antigens were

partially dissolved using overnight agitation with 0.2% Tween 20 in phosphate buffered saline (PBS) and the non-particulate fraction was analyzed according to Spencer and co-workers [16].

### 2.5. Detection of antigen-specific IgA

To detect and measure the salivary IgA levels specific to administered bacterial antigens, a novel enzyme immuno-assay was developed in microplates. Briefly, 5 μl of undiluted saliva were added to 5 μl micrograms of antigen powder in 0.05 ml of saline with Tween 20 and BSA. The incubation was carried out for 60 min at room temperature in a 96 well V buttomed microplate under agitation. After a washing cycle by centrifugation, a 1:3000 dilution of peroxidase-labelled anti-IgA in saline supplemented with Tween 20 (0.5%) and Bovine Serum Albumin (BSA, 3% w/v) was added and let to react for 60 min at room temperature. The unbound antibodies were removed from the particulate antigen by centrifugation. Finally, the peroxidase substrate (Sigma FAST OPD tablets), ready for the use, was added and let to react for a period ranging from 10 to 15 min. The reaction were then stopped using 2 N HCl in water and the supernatant was aspirated and transferred to a flat bottomed ELISA microplate for adsorbance at 450 nm reading. This was carried out using an automated microplate ELISA reader connected to a PC. ELISA results were calculated using Arbitrary Units (AU) by dividing, for each antigen, the adsorbance value of the proper test with the value obtained using an Ig negative control, such as fetal bovine serum (FBS). Rabbit specific antisera were used as positive controls.

### 2.6. Statistical analysis

The identification of patients characterized by the presence of a specific antibody mediated immune-response is strictly related to the definition of a cut-off value for the EIA used. Unfortunately, the unavailability of reference tests, which accurately quantitate the presence of anti-microbe antibodies in the salivary fluid, was the limiting factor to define this population of patients, the so-called negative control. In addition, a weak but consistently present cross-immunoreaction against microbial antigens was observed in virtually all analyzed samples. This low-affinity binding may be related to the highly homogeneous antigenic patterns of bacterial isolated in the respiratory airways. For this reason, a normal and symmetric distribution of observed results cannot be expected. On the contrary, a clear asymmetric distribution, caused by the absence of completely 'negative' results was hypothesized. For this reason, the distribution of all tested samples was calculated on the basis of AU results and two cut-off values were identified. These values were defined as the absorbance values within which 90 and 95% of tested samples were comprised. Of course, samples higher than these values were considered positive. The non-parametric chi-square ($\chi^2$) test was used for the comparisons of frequency.

## 3. Results

### 3.1. Analytical characteristics of the in vitro assay

To evaluate the occurrence of a natural immunization directed to surface antigens of a panel of bacteria, a novel assay was developed. Thus, the analytical characteristics of this novel approach were validated. The purity of the bacterial strains used was performed using routine identification tests in samples collected during bulk cultures. The antigenicity of different particulate preparations was documented using rocket electrophoresis on partially solubilized antigen from the particulate preparations (not shown). The characteristics of the novel enzyme immunoassay were defined using routine validation procedures. All tests were performed in triplicate, the coefficient of variation of intra-day reproducibility being 8.5%, and that of between day was 11.4%. Dilutions of both the antigens and antisera (or salivary fluids) resulted in a progressive reduction of the final absorbance values. On these bases, the assay was considered both specific and reproducible and was then used to analyze the presence of specific anti-bacterial IgA in saliva.

### 3.2. Analysis of the occurrence of anti-bacteria IgA in salivary fluids of untreated patients

Thirty-four samples, 21 from the adult and 13 from the children groups, were analyzed for the presence of specific anti-SA, SPy, KP, HI, MC and SPn antibodies. As expected, the distribution of all results was highly asymmetric (Fig. 1) and the use of the two cut-off values allowed the identification of salivary fluid samples characterized by the presence of anti-microbe IgA. Using this approach, a positive signal (i.e. AU values higher than the 90 and 95% of all tested sera) were observed in a fraction of patients. Of note, these cut-off values corresponded to a values ranging from 2 to 3 AU for the different antigens. To define the frequency of subjects secreting specific anti-microbe antibodies, the whole population was firstly studied, then the two subgroups, adults and children, were analyzed (Table 1). This analysis allowed the description of the percentage of patients (both adults and children) expressing IgA antibodies against a given antigen. As shown, in the whole population, only 29 and 24% of subjects had IgA directed, respectively, to KP and SA. The immune-response against other microbes was detectable in a

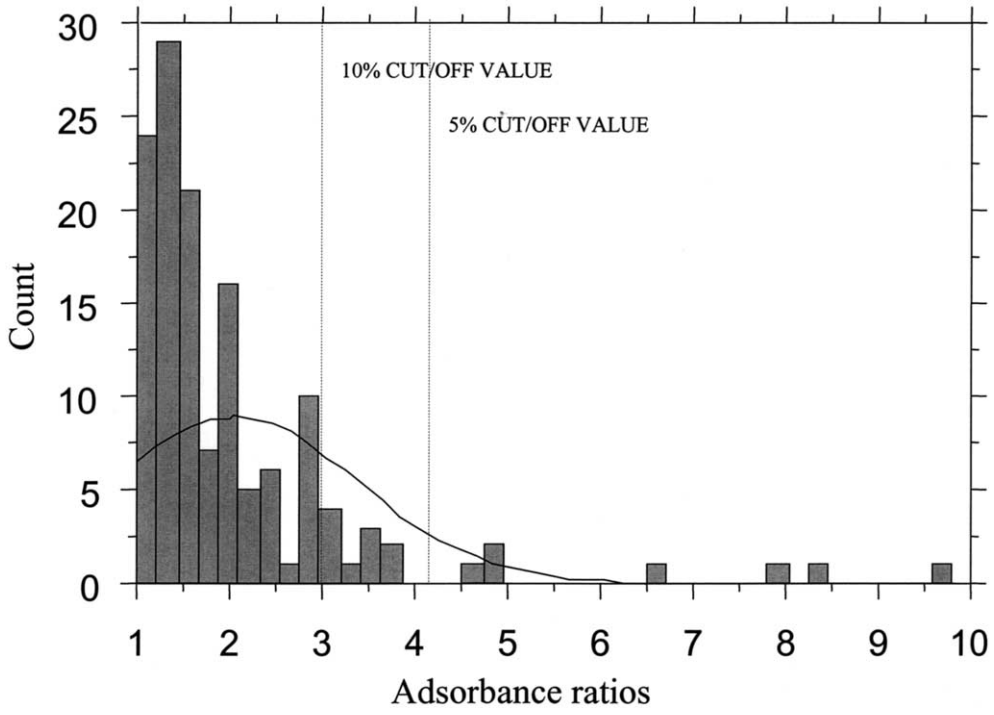


Fig. 1. Distribution of overall results obtained by analyzing specific IgA secretion. On the horizontal axis, the AU calculated on the adsorbance ratios; on the vertical axis, the count of samples within a given AU range.

small population ranging from 12 to 15% of all subjects studied. When the cut-off values was increased, in order to identify subjects with a high titer of specific IgA, these proportions were further reduced. When the two study populations were evaluated separately, we found that children expressed IgA titers against microbes at a very low frequency. Indeed, only KP raised a specific immune-response in 23% of tested young patients. In contrast, the presence of specific anti-microbes IgA was relatively more consistent in adults: only HI showed a low (14%) frequency of specific IgA response, while the other tested bacterial antigens were positive in 19–33% of tested individuals. These results are thus consistent with a poor frequency of specific anti-microbe antibodies in children and an only slightly higher proportion in adults. From a statistical point of view, the two populations were significantly different ($\chi^2 = 4.2$; $P =$ n.s. for the null hypothesis).

Using the same data, the frequency of specific IgA directed to SA, SPy, KP, HI, MC and SPn within the same patient was then studied (Fig. 2). This is particularly important to accurately define the more frequent panel of specific IgA expression in patients with a history of recurrent respiratory infections. Table 2 summarizes the results of this analysis and clearly shows that the presence of high titer antibodies directed to bacterial antigens is rare, particularly in children. Only one-third of the patients had an immune-response directed to one or two microbes, these immune reactions being characterized by low antibody titers. Finally, only a very small number of patients (four adults) have a strong immune-response directed to four different

Table 1
Counts (and frequency) of patients expressing specific anti-microbe antibodies

| Cut-off | Whole population ($n = 34$) | | Adults ($n = 21$) | | Children ($n = 13$) | |
|---|---|---|---|---|---|---|
| | 10% | 5% | 10% | 5% | 10% | 5% |
| KP | 10 (29) | 3 (9) | 7 (33) | 2 (10) | 3 (23) | 1 (8) |
| SA | 8 (24) | 2 (6) | 7 (33) | 2 (10) | 1 (8) | 0 (0) |
| SP | 4 (12) | 0 (0) | 4 (19) | 0 (0) | 0 (0) | 0 (0) |
| NC | 5 (15) | 2 (6) | 4 (19) | 2 (10) | 1 (8) | 0 (0) |
| HI | 4 (12) | 3 (9) | 3 (14) | 2 (10) | 1 (8) | 1 (8) |
| DP | 4 (12) | 2 (6) | 4 (19) | 2 (10) | 0 (0) | 0 (0) |

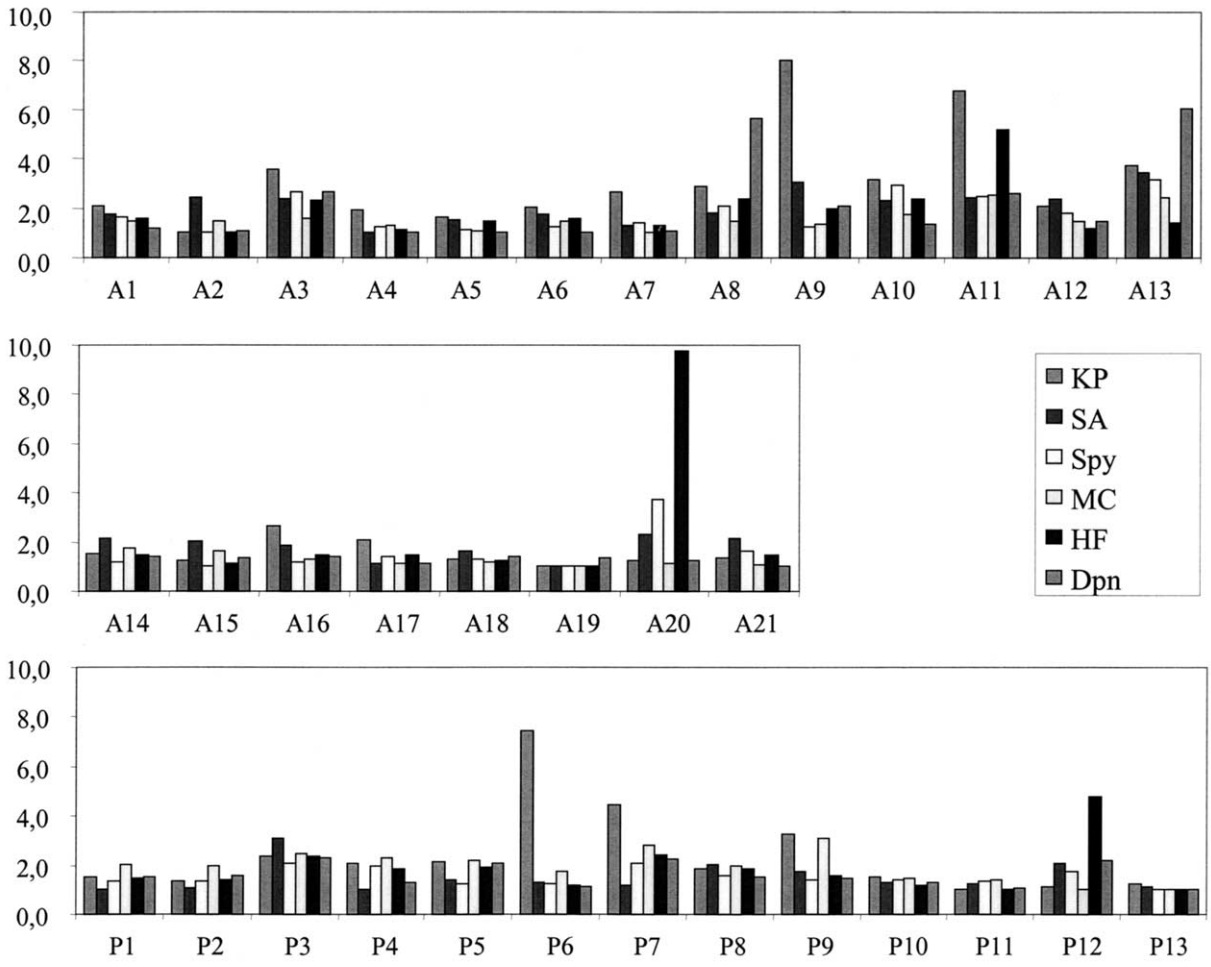


Fig. 2. Distribution of different immuno-reactivities against bacterial antigens at a single patient level. On the horizontal axis, the different patients analyzed for the six bacterial antigens. On the vertical axis, calculated AU for each antigen.

microbes. In children, the frequency of positive samples is even lower and no one pediatric patients was observed to have a strong IgA-mediated immune response directed to more than one respiratory bacteria.

### *3.3. Documentation of the increase of specific anti-bacteria IgA in healthy volunteers treated with a polyvalent bacterial mechanical lysate*

In the presence of a reduced frequency of naturally induced antibodies in salivary fluids in patients with recurred respiratory infections, a defect in the sensitivity of the enzyme-immunoassay used should be ruled out. For this reason, a group of ten healthy donors were treated with a polyvalent bacterial mechanical lysate (Ismigen®) for 10 days. A salivary sample was obtained before the beginning of the study and after 20 days from the end of the treatment. Eight out of ten treated volunteers experienced a significant enhancement of the concentration of salivary IgA specific for different bacteria. Along this line, KP was the strongest antigen (all but one subjects had anti-KP antibodies after one

Table 2
Counts (and frequency) of patients expressing one or more specific IgA antibodies

| | Number of positive IgA (%) | 6 | 5 | 4 | 3 | 2 | 1 | 0 |
|---|---|---|---|---|---|---|---|---|
| Overall | 10 | 0 (0) | 4 (12) | 0 (0) | 0 (0) | 4 (12) | 7 (21) | 19 (56) |
| $n = 34$ | 5 | 0 (0) | 0 (0) | 0 (0) | 2 (6) | 1 (3) | 4 (12) | 27 (79) |
| Adults | 10 | 0 (0) | 4 (19) | 0 (0) | 0 (0) | 3 (14) | 3 (14) | 11 (52) |
| $n = 21$ | 5 | 0 (0) | 0 (0) | 0 (0) | 2 (10) | 1 (5) | 2 (10) | 16 (76) |
| Children | 10 | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 1 (8) | 4 (31) | 8 (62) |
| $n = 13$ | 5 | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 2 (15) | 11 (85) |

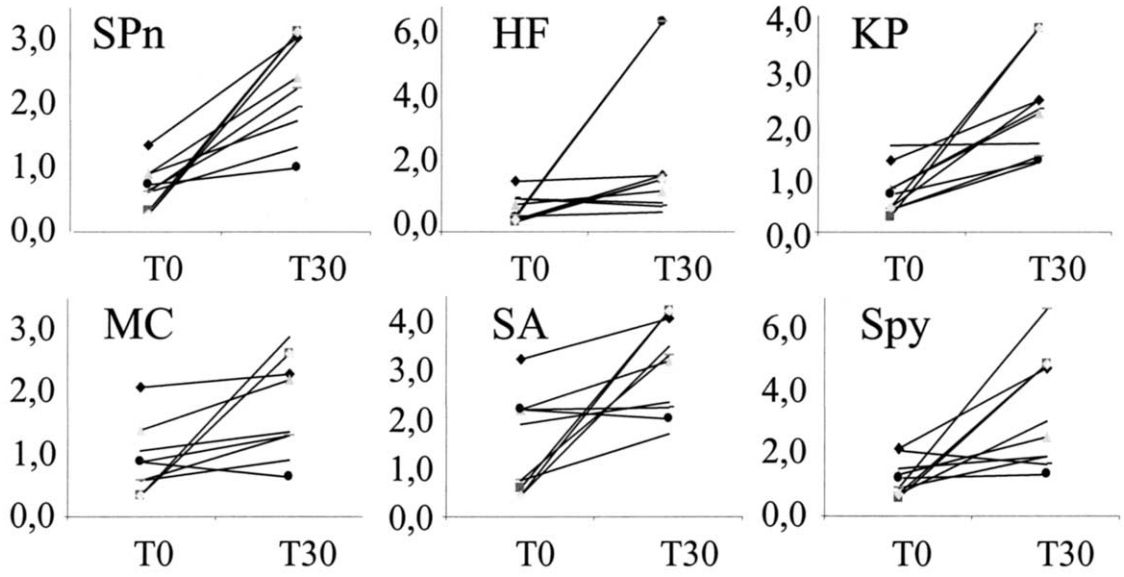


Fig. 3. Increase of the anti-bacterial titers in AU for the different microbes in the group of ten healthy donors treated with the polyvalent bacterial mechanical lysate. On the horizontal axis, day 0 and 30 of treatment. On the vertical axis, the calculated AU for each antigen.

cycle of treatment) and HF was the weakest, because only three volunteers experienced a significant rising of specific IgA (Fig. 3). Whatever the results obtained, these findings account for the sensitivity of the in vitro enzyme immunoassay used in this study.

## 4. Discussion

Any attempt to stimulate the immune response against bacteria, in order to enhance the concentration of antibody titers in the salivary fluid, requires the previous knowledge of the basal immune response in terms of naturally-raised antibody titers against the most frequent bacteria isolates.

In this study, using a novel immunoenzyme assay, we have analyzed the presence of IgA antibodies specific for six different pathogens of the respiratory tract, namely KP, SA, SPy, NC, HI, SPn. The assay had a good reproducibility and allowed an accurate follow-up of the specific anti-bacterial IgA titers in a group of volunteers treated with a polyvalent bacterial mechanical lysate. Nevertheless, what we also observed was that only a minor proportion of patients had a significant antibody titer naturally raised against these microbes. The distribution of positive results was apparently different in pediatric patients, where not only the number of positive subjects but also the number of specific antibody against each microbe is reduced. Of note, the same frequencies were observed in the basal samples obtained from the group of healthy volunteers treated with the polyvalent bacterial lysate.

All together, these date demonstrate that the frequency of IgA antibody titers in salivary fluids is low and subjects with a high titer are very infrequent even in the presence of a clinical history of recurrent respiratory tract infections. These antibodies have special features in the salivary fluid: for example, they have the capacity of opsonizing bacterial bodies, thus allowing their phagocytosis and subsequent killing. IgA are thus the main antibody isotype of mucosal secretions: they account for the first defense line against both bacterial and viral infections because of their activity against antigens recognized with high specificity and other antigens recognized with low specificity. Along this line, the results obtained in this work clearly demonstrated that in a population of patients with respiratory tract infections, even if collected during a asymptomatic period, the presence of an efficient immune-response against the potential pathogens is rare. This is particularly important in children, where our data have clearly shown that the percentages of positive patients are low.

Finally, the pathophysiological meaning of the results of the assay should be discussed. In clinical practice, the antibody titer against bacteria (such as streptococcus) is represented by anti-O streptolysin or anti-DNAse antibodies. These assays have a central meaning in the follow-up of patients with cardiac, renal or joint complications followed by a Spy infections. Nevertheless, these antibodies are directed to a extracellular toxin (the O streptolysin) and a intracellular enzyme (the DNAse). No titers are studied for antibodies that have the capability of neutralizing, by opsonizing bacterial bodies, the infectious microbes. The above described assay seems to be more specific in defining the actual locoregional defensive immune-response directed to relevant wall structures of infectious bacteria and could represent an actual estimate of a mechanism of defense.

These data may also have important consequences from a therapeutic point of view: indeed, it seems appropriate to try to stimulate, with a polyvalent bacterial vaccine, the oro-pharyngeal immune response against virtually all antigens in those patients with low antigen-specific IgA production, to evaluate whether an enhancement of the loco regional immune response could significantly reduce the recurrences of respiratory infectious episodes.

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