

Titres of specific antibodies to poliovirus type 3 and tetanus toxoid in saliva and serum of children with recurrent upper respiratory tract infections

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A study of antibody levels (in saliva and blood) against common vaccine antigens was performed in a population of 32 children suffering from recurrent upper respiratory tract infections (URTI). None of the patients had primary or secondary immunodeficiency syndromes or other known predisposing factors for respiratory diseases. Titres of the isotype-specific antibodies immunoglobulin A (IgA), immunoglobulin M (IgM), and immunoglobulin G (IgG) against two vaccine antigens – poliovirus type 3 (P3) and tetanus toxoid (TT), a viral antigen and a bacterial antigen, respectively – were measured in unstimulated saliva and serum, both in patients and in 24 healthy children (controls), by using a standard enzyme-linked immunosorbent assay (ELISA). In addition, levels of total IgA and avidity of IgA antibodies to both P3 and TT in saliva were evaluated. No difference was found between patients and controls as to levels of total IgA, or specific IgA and IgM antibodies against both P3 and TT in saliva. Furthermore, the avidity of salivary IgA antibodies against the two antigens did not differ between the two populations. However, the average concentrations of saliva-specific IgG antibodies to both the viral and the bacterial antigen were significantly lower ($p < 0.01$ for P3 and $p < 0.05$ for TT, respectively) in saliva of children with recurrent URTI, whereas no difference was found in serum for any immunoglobulin isotype determined compared with healthy individuals. The results of the present study provide suggestive evidence for the existence of subtle IgG-restricted defects in antibody responses at the mucosal level, but not at the serum level, in some children with undue susceptibility to URTI.

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Recurrent upper respiratory tract infections (URTI) in children represent a common problem in general practice. Although no exact data regarding the frequency of the phenomenon are available, in developed countries approximately one out of 10–20 children, mainly of preschool age, is afflicted with frequent URTI. The etiological agents are usually common respiratory viruses (1), although in many cases unfavorable environmental conditions, i.e. crowding, pre-

cious day-care attendance or passive smoking, play a relevant role (2). Only a small proportion of children with recurrent URTI show other obvious predisposing factors, such as anatomical anomalies, nutritional defects or primary immunodeficiencies, the latter being represented most commonly as selective antibody deficiency syndromes (3,4). Nevertheless, some subtle immunological defects, i.e. subnormal immunoglobulin levels, impairment of granulocyte functions or

abnormal cytokine production, are common *in vitro* and *in vivo* findings (5,6). Those defects in most cases are transient and may be secondary to the infections themselves, but still the question remains as to whether some children are more susceptible to respiratory illnesses because of certain immunological defects.

Secretory immunoglobulins (sIg), consisting mainly of immunoglobulin A (IgA), but also of immunoglobulin M (IgM), are a major component in the mucosal defence system against bacteria and viruses (7,8). Both sIgA and sIgM are transferred into secretions by the addition of a glycoprotein, the secretory component (SC), which is the extra-mural part of a transmembrane epithelial protein functioning as a polymeric immunoglobulin receptor. Immunoglobulin G (IgG) antibodies are also present in considerable amounts in respiratory fluids and probably cooperate with sIgA and sIgM to attain immune exclusion at the upper respiratory tract level (7). The existence of a common mucosal immune system, where antigen exposure in local sites results in spreading of antigen-specific sIgA-producing immunocytes into extensive mucosal surfaces, has been evidenced (8). Saliva belongs to this common mucosal immune system, as the predominant immunoglobulin in saliva is sIgA and epithelial cells in the salivary glands express the specific receptor for polymeric IgA molecules (9).

Little is known about specific antibody responses against microbial antigens at the mucosal level in children with recurrent URTI. The present study was carried out to investigate antibody responses against two vaccine antigens – poliovirus type 3 (P3) (a viral antigen) and tetanus toxoid (TT) (a bacterial antigen) – in salivary secretions of children with recurrent URTI.

Patients and methods

Subjects

All children afflicted with recurrent URTI who were referred to our clinic during a recent winter period (September 1998–March 1999) were enrolled in the study. Patients were defined as having recurrent URTI if they had a history of more than nine URTI per year (defined as an episode of five or more days with cough and/or sore throat), at least two-thirds of which were accompanied with fever or required antibiotic treatment, or more than four episodes of acute sinusitis or otitis per year in the last 2 years.

After the first visit, an immunological investigation was carried out in each patient. This

investigation consisted of: delayed type hypersensitivity skin tests, a white cell differential count, lymphocyte phenotypic analysis, and immunoglobulin and IgG sub-class determination.

IgG sub-classes were measured by using nephelometry (Behring Diagnostics, Marburg, Germany), according to the manufacturer's instructions. Levels of IgG sub-classes above the 3rd percentile, according to the age-appropriate values published by Plebani et al., were considered normal (10).

Lymphocyte phenotypes in blood were characterized using a fluorescence-activated cell sorter (FACScan flow cytometer; Becton-Dickinson, Milano, Italy) and direct conjugate two-color labeled monoclonal antibodies (Becton-Dickinson). The expression of the following marker antigens was determined: CD3 (T cells), CD4 (T-helper cells), CD8 (T-cytotoxic cells), CD16 (natural killer [NK] cells), and CD20 (B cells).

Patients with recognized immunodeficiency syndromes and/or the presence of any abnormality (such as low levels of NK cells, subnormal immunoglobulin levels, etc.) in one or more immunological parameters, were excluded from the study. Other inclusion criteria for further recruitment were: (i) absence of systemic diseases or any known factor, such as iron deficiency, malnutrition, etc., influencing the host defence (on physical examination and other adjunctive laboratory investigations performed at the physician's discretion); (ii) negative history about relevant risk factors for recurrent URTI in the patient's environment (i.e. passive smoking, poor socio-economical conditions or crowding, defined as more than one person resident per room in the home).

Patients were defined as atopic if they had increased levels of total or specific immunoglobulin E (IgE) in serum and/or hay-fever, recurrent wheezing, atopic dermatitis or urticaria in their history.

Thirty-two children (19 males and 13 females), mean age 4.9 years (range 24 months to 13 years) were eligible for the study.

Twenty-four healthy children, age and gender matched, with no history of recurrent URTI and normal immune parameters, were used as controls. Most were children or relatives of hospital employees.

Demographic, clinical, and main laboratory characteristics of the patients and controls are shown in Table 1.

Unstimulated saliva samples were obtained over a fixed time-period of 15 min, 2 h after breakfast, by gentle suction from the buccal sulci

Table 1. Demographic, clinical, and immunological characteristics of the subjects studied

	Patients (n=32)	Healthy control subjects (n=24)
Age (years; mean $\pm$ SEM) (range)	4.90 $\pm$ 0.3 (2–13)	4.80 $\pm$ 0.4 (1.9–13)
Male/female ratio	1.4	1.4
Atopic history (%)	25	25
Immunoglobulin levels in serum*		
IgG	10.23 $\pm$ 0.43	10.32 $\pm$ 0.35
IgA	0.83 $\pm$ 0.06	0.88 $\pm$ 0.07
IgM	1.54 $\pm$ 0.10	1.20 $\pm$ 0.06
IgG ₁	6.82 $\pm$ 0.39	6.65 $\pm$ 0.39
IgG ₂	1.82 $\pm$ 0.18	1.78 $\pm$ 0.17
IgG ₃	0.64 $\pm$ 0.07	0.63 $\pm$ 0.06
IgG ₄	0.48 $\pm$ 0.09	0.32 $\pm$ 0.09
Lymphocytes in blood ($\times 10^9/l$)	3.12 $\pm$ 0.17	2.97 $\pm$ 0.17
Proportion of lymphocyte phenotypes†		
CD3	74.12 $\pm$ 0.83	73.50 $\pm$ 0.87
CD4	43.80 $\pm$ 0.79	45.30 $\pm$ 1.11
CD8	24.30 $\pm$ 0.73	22.40 $\pm$ 0.83
CD16	11.00 $\pm$ 0.32	10.10 $\pm$ 0.43
CD20	10.50 $\pm$ 0.40	9.90 $\pm$ 0.49

*g/l; mean $\pm$ SEM.†%; mean $\pm$ SEM.

using a round-tip sterile glass pipette with a capillary lumen. Care was taken to avoid trauma of the oral mucosa, which could result in contamination of the samples with blood. Soon after collection, cell debris in the saliva was removed by a 10-min centrifugation at 4000 g/min, and the supernatants were stored at -20°C in sterile plastic tubes until used.

Blood samples were also obtained from each individual by venepuncture and the sera was stored at -20°C , as described above. All patients and controls had been vaccinated with live attenuated poliovirus type 1–3 vaccine and *Clostridium tetani*-inactivated toxin, according to the national vaccine schedule.

Informed consent was obtained from all parents, and the study was approved by the Institutional Review Board.

Total IgA measurement in saliva

Total IgA in saliva was determined by radial immunodiffusion using commercial agarose plates, low-range, incorporating a sheep anti-human IgA, α -chain specific (Behring Diagnostics), according to the manufacturer's instructions. IgA levels were calculated by multiplying the obtained values by 3.25, according to Brandtzaeg et al. (11), in order to correct for physicochemical differences between serum and secretory IgA immunoglobulins.

Measurement of specific IgA, IgG, and IgM antibodies against P3 and TT in saliva and serum

Specific antibodies against P3 and TT were determined by using a standard enzyme-linked

immunosorbent assay (ELISA), as previously described (12). Briefly, 96-well polystyrene microtitre plates (Nunc-Immunoplate 1; Nunc, Roskilde, Denmark) were coated with 100 μl of TT (kindly provided by Dr M. Nitti, Istituto Sieroterapico e Vaccinogeno Berna, Como, Italy) and P3 (a kind gift of Dr A. van Wezel, Rijksinstituut voor Volksgezondheid en Milieuhygiene, Bilthoven, the Netherlands) diluted with phosphate-buffered saline (PBS), pH 7.2, and incubated overnight in a moist chamber at 5°C . After three washes with PBS containing 0.05% Tween-20 (PBS-T), the samples were diluted in 10-fold serial dilutions with PBS-T, starting from 1/10 and 1/100 for saliva and sera, respectively, and then transferred to microplate wells in duplicate. (To minimize the influence of intra-assay variations, samples from patients and controls were always run on the same microtitre plate.) The plates were then incubated at 5°C for 3 h. After washing with PBS-T, the wells were filled with 100 μl of alkaline phosphatase-conjugated affinity-purified rabbit anti-human immunoglobulin (α -, γ - or μ -chain specific) (Dako, Milano, Italy) diluted 1:1000 with PBS-T, and incubated overnight at 5°C . After washing, 100 μl of 0.1% wt/vol crystalline *p*-nitrophenyl phosphate disodium (Sigma, Milano, Italy) in 1 M diethanolamine containing 0.002 M MgCl_2 (pH 9.8) was added to each well and the plates were incubated in the dark at room temperature for a maximum of 100 min. Absorbances were read at a wavelength of 405 nm in a Titertek Multiskan MK II (Flow Laboratories, Settimo Milanese, Italy). A pool of human saliva and sera with high titres of specific antibodies against both antigens was used as a reference. Results were

Table 2. Serum antibody levels – immunoglobulin A (IgA), immunoglobulin G (IgG), and immunoglobulin M (IgM) – to poliovirus type 3 (P3) and tetanus toxoid (TT) in the subjects studied

	Patients	Healthy control subjects	p-value
P3			
IgA	88 (50–121)	102 (65–155)	0.211
IgG	91 (61–107)	91 (58–119)	0.607
IgM	101 (73–160)	91 (75–149)	0.685
TT			
IgA	97 (81–144)	86 (69–98)	0.147
IgG	93 (58–121)	87 (69–104)	0.523
IgM	96 (55–125)	98 (84–139)	0.246

Results are expressed as % of reference: median $\pm$ inter-quartile range (IQR).

calculated from the optimal dilution giving an absorbance in the linear part of the reading curve of the spectrophotometer and expressed as per cent of the reference.

Reproducibility was always less than $\pm 10\%$ between tests and $\pm 5\%$ within tests.

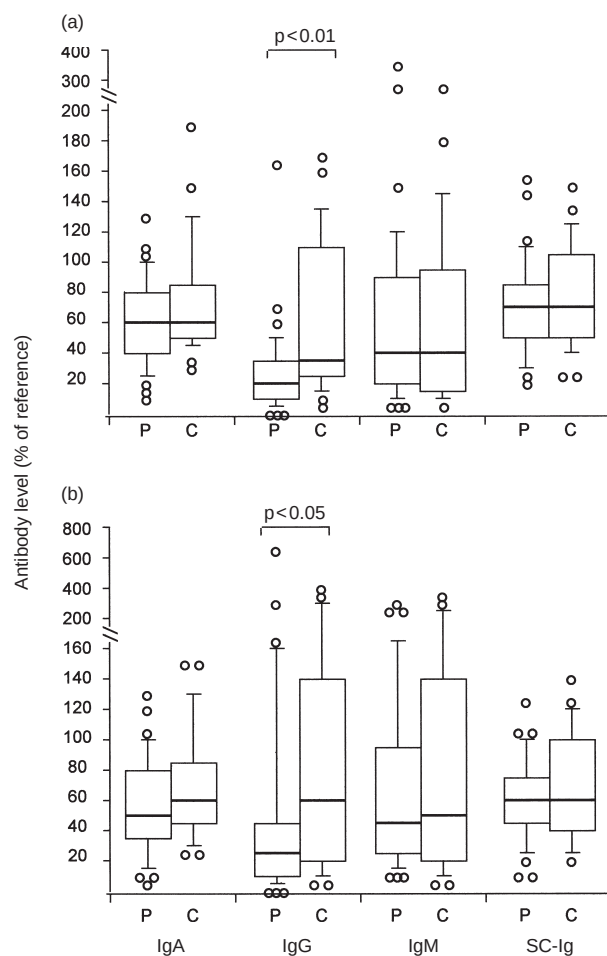


Fig. 1. Salivary antibody levels [immunoglobulin A (IgA), immunoglobulin G (IgG), immunoglobulin M (IgM), and secretory component (SC)-coupled immunoglobulins] to poliovirus type 3 (A) and tetanus toxoid (B) in children with recurrent upper respiratory tract infections (URTI) (P) and healthy control subjects (C). The median is the line in each box; box limits represent inter-quartile range (IQR). Whiskers extend to the 10th and 90th percentiles. Values above the 90th percentile and below the 10th percentile are plotted separately.

Measurement of secretory antibodies against P3 and TT in saliva

Secretory immunoglobulins to P3 and TT were measured in saliva by using a standard ELISA, as described above, but with alkaline phosphatase-conjugated affinity-purified sheep anti-serum, human-SC specific (The Binding Site, Birmingham, UK), diluted 1:500 in PBS-T. Optical densities were read at a wavelength of 405 nm after incubation for 100 min, and the results expressed as per cent of the reference.

Avidity of IgA

The avidity of IgA antibodies to P3 and TT in saliva was determined by using the thiocyanate-elution enzyme immunoassay, as previously described (13). This method, described by Pullen et al., is based upon the capacity of the thiocyanate caotropic ion to elute antibodies from solid-phase immobilized antigens (14). Avidity is therefore expressed as the molarity of thiocyanate required to elute 50% of the antigen-bound IgA antibody under conditions of antigen excess.

Statistics

Tests for normality (Shapiro-Wilk) were performed. As the values, in general, did not have a normal distribution, the statistical analysis was carried out using non-parametric tests (Mann-Whitney *U*-test and Spearman's rank correlation test: Primer[®], Application Software, Ver. 4.02) (15). Only two-tailed tests were used. A p-value of less than 0.05 was considered significant. Results are presented as median $\pm$ inter-quartile range (IQR).

Results

The concentration of total IgA in saliva did not differ significantly in children with recurrent URTI as compared with non-affected subjects 0.07 g/l (25–75%: 0.01–0.14 g/l) in patients and 0.08 g/l (25–75%: 0.02–0.14 g/l) in controls, difference NS. The titres of specific antibodies in saliva and serum are shown in Fig. 1 and Table 2, respectively. No significant difference was found in the levels of IgA antibodies to P3 and TT in saliva and serum between the groups. Nevertheless, a trend towards lower levels of salivary IgA antibodies was observed in the patients as compared with the non-affected subjects. A good correlation was found between specific IgA antibodies against both antigens and the levels of SC-carrying antibodies in saliva, confirming that the IgA immunoglobulins in saliva were mostly of secretory origin (correlation coefficient $r_s=0.668$, $p < 0.001$, and 0.706 , $p < 0.001$, for P3 and TT, respectively).

The avidity of IgA antibodies to the viral or the bacterial antigen did not differ between the patients and the controls: 1.0 (25–75%: 0.8–1.5) vs. 1.0 (25–75%: 0.7–1.3), respectively, for anti-P3 antibodies, difference NS; 0.6 (25–75%: 0.4–1.0) vs. 0.5 (25–75%: 0.4–0.9) for anti-TT antibodies, difference NS.

The level of IgG antibodies to both P3 and TT was significantly lower in the saliva of children with recurrent URTI compared with the healthy individuals: 19.5 (25 to 75%: 12–37) vs. 36 (25 to 75%: 22.5–110), respectively, for anti-P3 antibodies ($p < 0.01$) and 24 (25 to 75%: 12.5–47) vs. 59 (25 to 75%: 22–138.5) for anti-TT antibodies ($p < 0.05$). Those differences, however, were not reflected in serum where the level of IgG antibodies to both antigens did not differ between the patients and the controls. Comparable titres were observed for the two groups, both in saliva and in serum, for IgM antibodies.

Discussion

URTIs constitute by far the most common acute illnesses in children in developed countries. The lack of immunological experience at birth and an incomplete maturation of some host defence mechanisms may account for the increased susceptibility of infants and children to respiratory infections as compared to adults. On the other hand, the frequency of episodes seems to be heavily influenced by environmental factors, such as parental smoking, day-care attendance, and indoor or outdoor pollution (2,16). The importance of other factors, such as gender, family size,

or atopy, appears more questionable. In some children with a high frequency of URTI, subclinical trace element deficiency or primary immunodeficiency syndromes can also be detected (3,4). In other cases, however, this undue susceptibility to infections seems not to be related to any obvious predisposing factor. As an important role in the immune defence system of mucosal surfaces against both bacteria and viruses is played by secretory immunoglobulins (sIgA, but also sIgM), a relative defect of antibody responses at this level in children with recurrent URTI could be hypothesized. One major function of secretory immunoglobulins is to provide immune exclusion of micro-organisms, by neutralizing toxins and viruses and preventing bacterial adherence to epithelial surfaces (7,8). sIgA represents the main immunoglobulin in external body fluids and shows different structural properties from serum IgA, as it is present mainly in a dimeric form and is coupled with the SC, an 80 000 Da molecular weight glycoprotein of epithelial origin functioning as a polymeric immunoglobulin receptor.

sIgM is likewise associated with the SC and is actively transported into secretions from glandular epithelia, mainly in the absence of IgA. Immunochemical and immunohistochemical studies have also shown that the absolute majority of sIgA and sIgM in humans is produced locally from mucosal immunocytes (7,8,17,18). In the early 1970s, Craig & Cebra, shortly followed by many other investigators, demonstrated in animal models that the Peyer's patches are an important source of IgA-producing immunocytes for both the recipient's intestine and other mucosal tissues and glands (19). Since then, the existence of a common mucosal immune system, where committed lymphocytes originating in the gut-associated and bronchus-associated lymphoid tissues (the so-called GALT and BALT) migrate to other mucosal and exocrine glandular tissues, has been found both in animal models and in humans (8). Some evidence, however, suggests that a dichotomy exists in cellular migration between the upper airway and the intestine and that the BALT does not constitute a major source of sIgA immunocytes for the upper respiratory tract (7,20). Moreover, the existence of BALT in humans has been questioned (21). In fact, the lymphoid tissue of the Waldeyer's ring probably represents the most important lymphoepithelial tissue at the upper respiratory level. According to this hypothesis, sensitized B cells originate from the palatine tonsils or adenoids and migrate to other areas of the upper airways, including salivary and lacrimal glands, middle

ear, and nasal mucosa (7,20). Therefore, the analysis of antibody levels in salivary fluids could reasonably provide a good picture of immune responses in other areas of the upper respiratory tract. However, any measurement of antibody levels to common respiratory micro-organisms in infection-prone children, as compared with normal individuals, may be influenced by a different degree of antigen exposure. From this point of view, it may be more appropriate to measure titres of specific antibodies to vaccine antigens in order to explore the capacity for immune responses.

Decreased total sIgA has already been described in the saliva of children with atopy (22), adenoid hyperplasia, and otitis media with effusion (23). Furthermore, Banzoff et al. indicated a defective salivary IgA response against the respiratory syncytial virus in symptomatic allergic children at 1 year of age (24).

Our study showed that secretory immunity, as determined by measuring both total and specific sIgA to two vaccine antigens in saliva, was not impaired in children with recurrent URTI of unknown origin. Furthermore, the titre of the other partly secretory immunoglobulin in saliva, IgM, did not differ in the affected children and the healthy individuals. As the mean levels of serum IgE and a previous history of allergy were not different between patients and controls, an influence of factors related to the atopic/non-atopic status seems unlikely. However, the titres of specific IgG to both TT and P3 were significantly lower in salivary fluids of children with repeated URTI, but these differences were not detected in serum.

Antibodies against viral, bacterial, and fungal antigens have been detected in saliva (24–26). Unlike sIgA, which is actively secreted from glandular epithelia, the majority of IgG in saliva and respiratory fluids is mainly derived from a passive leakage through the mucosal surface (7). As a result, IgG levels in salivary fluids are dramatically lower than those of sIgA and also of IgG in serum (27). However, whether locally produced or passively transferred into secretions, IgG antibodies are thought to take part in immune exclusion in the upper respiratory tract (7). Furthermore, IgG immunocytes represent the most common immunoglobulin-bearing cell population in the tonsils, the nasopharyngeal adenoids, and the nasal mucosa beneath the surface epithelium (7,20,28). However, to date the exact role of IgG antibodies in the mucosal defence system is not fully understood. As bronchial and nasal fluids have a relatively low proteolytic activity it can be assumed that those

IgG could be protective. Moreover, tissue irritation resulting from microbial infections is thought to enhance the leakage of locally or serum-derived IgG antibodies across the respiratory mucosa (7).

Our results suggest the existence of a subtle underlying defect of the immune response at the mucosal level in a group of children with recurrent URTI of unknown origin. This defect seemed to be limited to IgG antibody responses, which have a somewhat uncertain role in the mucosal defence system. Moreover, the question of whether this defect is primary or just secondary to the infections still remains unresolved.

An impairment of antibody responses against capsulated bacteria, sometimes with normal levels of IgG and IgG sub-class immunoglobulins in serum, has been identified by several authors in some children afflicted by recurrent URTI (29,30). Most of these patients usually suffer from recurrent otitis and/or sinusitis, pneumonia or invasive bacterial infections. The occurrence of such a selective antibody deficiency in our population of children with recurrent URTI of unexplained origin cannot be ruled out, as we did not explore the patients' ability to mount adequate antibody responses against polysaccharide antigens. This hypothesis, however, seems unlikely, as only a minority of our patients were afflicted with recurrent sino-pulmonary infections and none had a history of severe bacterial infections. Moreover, in a study of Epstein et al., a low prevalence was found (6.5%) of selective deficiency of antibody responses to pneumococcal capsular antigens in a large population of children with recurrent respiratory infections and normal immunoglobulin levels (30).

Further studies are therefore required to demonstrate the possible clinical relevance of our preliminary findings. These should include long-term follow-up observations, as many children with recurrent URTI, whether or not afflicted with selective antibody deficiency, recover spontaneously with time (31).

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