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IgD in nasopharyngeal secretions and tonsils from otitis-prone children

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SUMMARY

Quantification of IgD was performed by ELISA in 180 plasma samples and 83 nasopharyngeal secretions (NPS) from children aged 2–162 months with varying degrees of recurrent otitis media. Furthermore, in 24 of the children the density of the IgD-immunocytes (IgD-cells) was calculated in immunoenzyme-stained cross-sections of their nasopharyngeal tonsils (NPT). Owing to a considerable variation of the IgD cell density throughout the NPT, a semi-quantitative counting system was applied. An irregular distribution of plasma IgD was observed during childhood and maximum levels were found between 48 and 72 months of age. However, an even distribution of plasma IgD was found among the four groups of children investigated. Based on calculations of the transudation of albumin from plasma to the NPS the amount of locally produced IgD in NPS (NPS-IgD (local)) was estimated to 88% (range 41–99%). Significantly higher levels of NPS-IgD (local) were found in otitis-prone children than in the other groups. Moreover, a positive correlation was calculated between levels of NPS-IgD (local) and NPT-IgD cell density, indicating that NPT, being the local lymphoepithelial tissue, also functions as an important source of NPS-IgD. NPS-IgD was not found to be associated with secretory component, indicating a passive transfer of IgD through the mucosal membranes. Our results support the hypothesis of an association between the occurrence of IgD in the mucosa and secretions of the upper respiratory tract with localized inflammatory events.

Keywords IgD nasopharyngeal tonsils nasopharynx acute otitis media secretory otitis media

INTRODUCTION

In mammals, immunoglobulin D (IgD) is expressed on the surface of virgin B lymphocytes (B cells) and usually disappears during further B cell differentiation and maturation (review by Cooper *et al.*, 1982; Jelinek & Lipsky, 1987) consistent with the isotype switching of the cell (Black *et al.*, 1978; Kuritani & Cooper, 1982). However, occasionally, human B cells mature into IgD-secreting immunocytes (IgD-cells) and of special interest is the observed variation in the distribution of such cells (Rowe, Crabbe & Turner, 1968; Brandtzaeg *et al.*, 1979). An increased number of IgD cells has been found in sites of lacrimal, nasal, salivary and mammary glands compared with the only occasional occurrence in fundic, jejunal and colonic areas (Brandtzaeg *et al.*, 1979; Korsrud & Brandtzaeg, 1980a). Moreover, nasopharyngeal and palatine tonsils contain far more IgD-cells than do spleen and lymph-nodes (Rowe, Crabbe & Turner, 1968; Brandtzaeg, Surjan & Berdal, 1978; Korsrud & Brandtzaeg, 1980b). In addition, a more pronounced variation in the distribution of glandular IgD cells has been observed in patients with selective IgA deficiency (Brandtzaeg *et al.*, 1979).

However, limited clinical information is available on the quantitative and functional aspects of IgD in secretions and lymphoid tissue from the upper respiratory tract (Plebani *et al.*, 1983; Sørensen, 1983; Brandtzaeg *et al.*, 1987). A relative increase of the IgD cell density has been found in palatine tonsils from patients with recurrent tonsillitis compared with tonsils from healthy individuals (Surjan, Brandtzaeg & Berdal, 1978). Also, the accumulation of IgD cells in nasal mucosa of patients with selective IgA deficiency seems to be associated with recurrent infections at the mucosal level (Brandtzaeg *et al.*, 1987). Previously, we found high levels of IgD in middle ear effusions (MEE) from children with recurrent otitis media and the data substantiated a considerable local production of IgD (Sørensen, 1983). Apparently, the occurrence of IgD in the mucosal membranes and secretions of the upper respiratory tract is associated with localized recurrent inflammatory events. Supporting evidence for this hypothesis is given in the present study in which we describe quantitative aspects of IgD in plasma, MEE, saliva, nasopharyngeal secretions and tonsils from children with varying degrees of recurrent infections of the upper respiratory tract.

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MATERIALS AND METHODS

Plasma samples

These were obtained from 112 boys and 68 girls, aged 2–162 months. The 180 children were classified into the following four groups:

(1) 42 children, median age 47 months (interquartile range (Iqr) 24–55), having experienced more than six episodes of acute purulent otitis media (AOM) during the preceding 1–2 years, denoted otitis-prone children (OP group). In addition, all children had secretory otitis media (SOM) 3–6 months before hospitalization.

(2) 50 children, median age 57 months (Iqr: 48–66), comparable to the group of OP children except that they had less than five recurrent episodes of AOM 1–2 years before sampling (rAOM group).

(3) 51 children, median age 59 months (Iqr: 41–70) having SOM for at least 3–6 months before the examination (SOM). Children with SOM most often appear without clinical signs of acute illness, except for recurrent common colds and hearing disability. None of the children in this group had previously suffered from AOM. The diagnosis of SOM was confirmed by myringotomy.

(4) 37 healthy children, median age 63 months (Iqr: 47–109). Of these, 22 children had operations for hernia, phimosis, or protruding ears and 15 children had adenoidectomy because of enlarged nasopharyngeal tonsils, resulting in snoring as the only symptom. None of these children had previous episodes of AOM or SOM.

Common to all 180 children was the fact that none of them had AOM or received any antibiotics within the 3 weeks before hospitalization. Normal levels of plasma IgA were found in all children investigated (Sørensen & Nielsen, 1988).

Nasopharyngeal secretions (NPS)

These were collected from 83 of the 180 children, aged 14–128 months. According to the criteria above, the 83 children could be classified as 19 OP, 29 rAOM, 29 SOM and six healthy children. The latter group underwent adenoidectomy because of mechanical obstruction of the nasopharynx. While the child was still under general anaesthesia, the secretion pool located inferior and lateral to the nasopharyngeal tonsils was sucked up directly under visual guidance, immediately frozen at −80°C, and processed as described by Sørensen (1982; 1983). Cord sera were obtained from 44 healthy babies, and unstimulated whole-saliva from 119 healthy 4-year-old children was collected in ice-chilled polystyrene tubes, as described by Sørensen (1982).

Immunoassay

Quantification of IgD in plasma, cord sera and secretion samples was performed by ELISA, as reported by Sørensen (1983). Briefly, gamma-irradiated polystyrene microtest plates (NUNC AS, Copenhagen) were incubated overnight at 4°C with rabbit Ig specific for heavy-chains of human IgD (Dakopatts A/S, Denmark). A plasma standard, containing 24 mg/l of IgD as compared with the British reference standard 67/37, was used and seven serial dilutions were applied in quadruplicates on each plate. Test samples were applied in duplicates. The capture antibody conjugated with horseradish peroxidase was used as the detector antibody (Dakopatts A/S, Denmark). The monospecificity of the rabbit antisera used was proved by means of crossed immunoelectrophoresis techniques. IgD standard curves (one for each plate) were computed by means of a curve-fitting procedure (Sørensen, 1982) and the analytical range was 96–1·5 μg/l. Curves obtained by serial dilutions of both sera and secretion samples were found to be parallel to corresponding curves of the plasma standard. The putative association of NPS-IgD with secretory component (SC) was sought, illustrated by the following ELISA: (1) anti-δ; (2) NPS-IgD proteins; (3) anti-SC conjugated with horseradish peroxidase. However, in 20 NPS with high levels of IgD, the anti-SC reactivity was indistinguishable from the background absorbance. The albumin content in NPS and in paired plasma samples was measured by means of routine rocket immunoelectrophoresis. The concentration ratio of NPS-albumin/plasma-albumin was used for calculation of the amount of NPS-IgD transudated from plasma (Donovan *et al.*, 1970; Table 1).

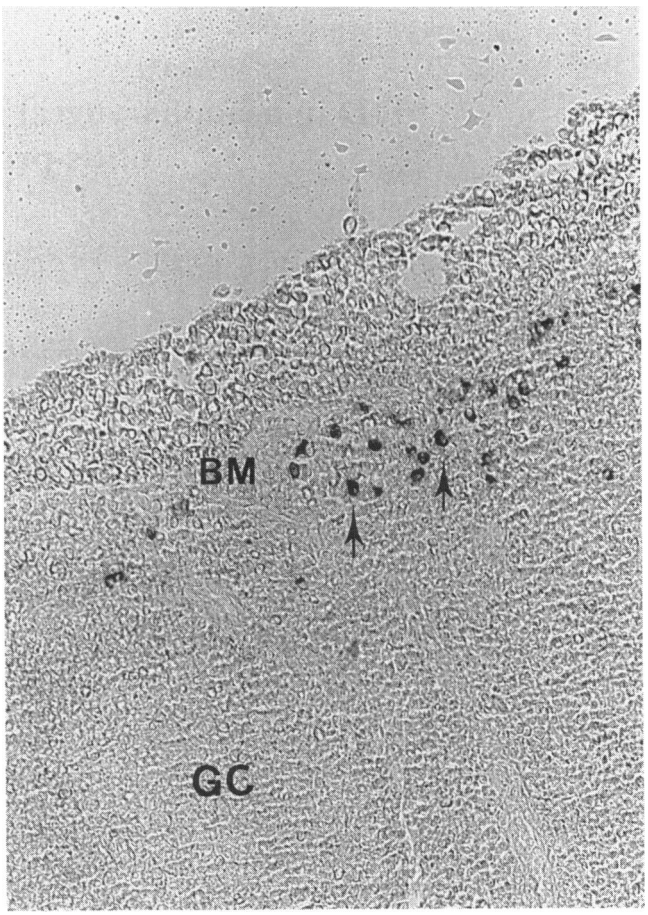

Fig. 1. Immunoenzyme staining (peroxidase labelling without counter-staining) of IgD-cells in cross sections of nasopharyngeal tonsil from a child with recurrent acute otitis media. Irregular distribution of IgD cells (arrows), mainly located in the subepithelial layer of the extrafollicular area. Basement membrane (BM) and germinal centre (GC) are indicated (×250).

The intra-assay variation was estimated in 52 NPS and 66 plasma samples randomly selected. In NPS with IgD > 13 mg/l the coefficient of variance (CV) was 9·2% and for NPS IgD ≤ 13 mg/l, the corresponding CV was 3·7%. In plasma IgD > 15 mg/l, the CV was 8·9% and for plasma IgD ≤ 15 mg/l the CV was 7·5%. The inter-assay variation was estimated in five NPS

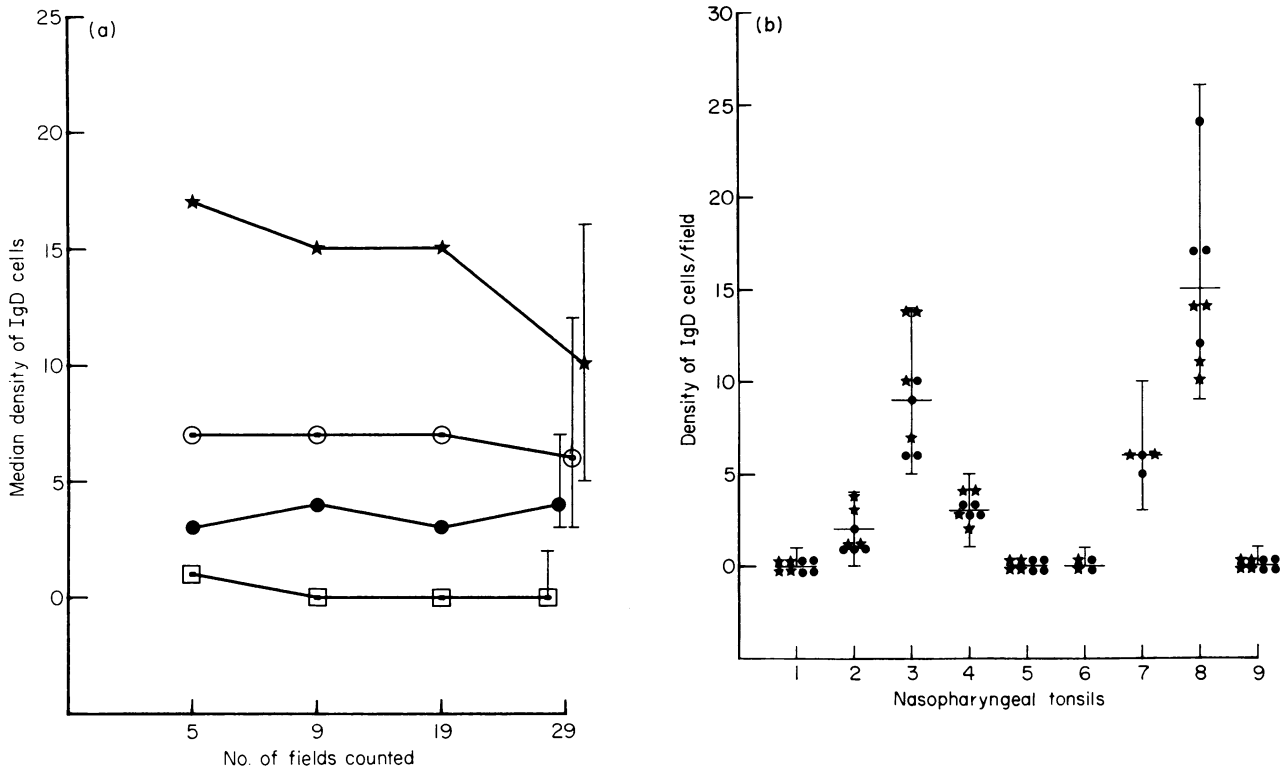

Fig. 2. Methodological aspects of enumeration of IgD-cells in sections of nasopharyngeal tonsils (NPT). (a) The median density of IgD-cells is expressed as a function of the number of fields counted in four different NPT. The interquartile ranges are given per 29 fields counted (vertical lines) and illustrate the necessity of counting 29 fields in order to obtain a reliable estimate of the median density per section. (b) The intra-individual variation of IgD-cell density was evaluated in NPT from nine children. In each NPT, the median IgD-cell density was calculated per 30 fields counted in eight different localities (only four in cases 6 and 7) distributed superiorly (*) as well as inferiorly (●). The overall median densities and interquartile ranges are illustrated by horizontal and vertical lines, respectively.

(range 6·2–75·3 mg/l of IgD) and five plasma samples (range 3·3–58·3 mg/l of IgD) and measured over a period of 7 days. The CV for NPS-IgD was 3·3% (range 1·1–5·4%) and the corresponding CV for plasma IgD was 4·6% (range 1·6–6·8%).

Immunohistochemistry

Nasopharyngeal tonsils (NPT) were removed from nine children because of rAOM and SOM. The tonsils were excised with great care in order to preserve the tissue *in toto*. From each NPT eight different pieces of tissue (about 5 × 5 × 5 mm) were cut out to evaluate the superior–inferior intraindividual variation of the IgD-cell density (Fig. 2b). Immediately after removal the tissue specimens were placed in cold circulating PBS for 24 h before ethanol-fixation and paraffin-embedding, as described by Brandtzaeg (1974). Histological sections were cut at 5 μm and incubated for 30 min with rabbit Ig, specific for human IgD (Dakopatts A/S, Denmark) using an indirect enzyme-immunohistochemical technique (Clausen *et al.*, 1979) (Fig. 1). In some NPT, no positive IgD cells could be detected, but adjacent sections always showed normal reaction with rabbit Ig, specific for human IgA, IgG and IgM, respectively. Enumeration of cytoplasmic-positive IgD cells was done in high-power fields (HPF, ×400) and only tissue sections with intact basement membranes were used. The density of the IgD cells was evaluated in HPF defined by the subepithelial area obtained by setting the top of the outer frame just beneath the basement membrane (Fig. 1). The median density of IgD cells was calculated per 5, 9, 19, and 29 randomly selected fields (Fig. 2a). In order to evaluate the correlation between NPT-IgD cell density and levels of NPS-IgD, 24 consecutive children were investigated further. All children underwent adenoidectomy and myringotomy for insertion of ventilating tubes because of rAOM and SOM. The middle ear effusions (MEE) were aspirated and immediately frozen before adding PBS. Quantification of IgD (MEE-IgD) was performed by ELISA (Sørensen, 1983). Samples of plasma, NPS and NPT were collected and processed as described above and the enumeration of IgD-cells was done according to the principles just mentioned. However, due to a notable intra-individual variation throughout the NPT (Fig. 2b), the density of IgD cells was expressed semi-quantitatively as follows: + = 0 IgD cells/field; + + = 1–5 IgD cells/field; + + + = > 5 IgD cells/field. In all tissue specimens, the median densities were based on counting of 30 fields.

Statistical methods

Groups of data were tested for normality by means of the Kolmogorov–Smirnov one-sample, goodness-of-fit test (Siegel, 1956). The distributions of NPS-IgD especially were considered to be non-Gaussian and nonparametric statistical analyses were therefore used (Siegel, 1956). Probability (P) $\leq 0{\cdot}05$ was chosen as the level of significance (two-tailed).

RESULTS

Levels of IgD in cord sera and plasma samples are shown in

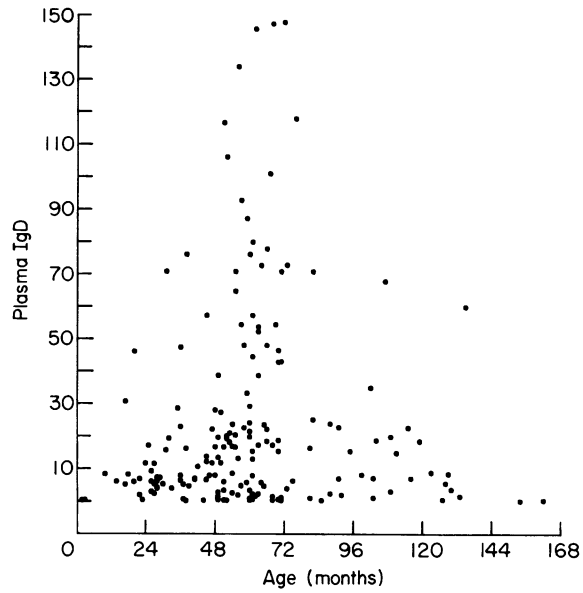

Fig. 3. Levels of plasma IgD in 180 children as a function of age. Rho. (Spearman correlation coefficient) = 0·13; $P > 0·1$.

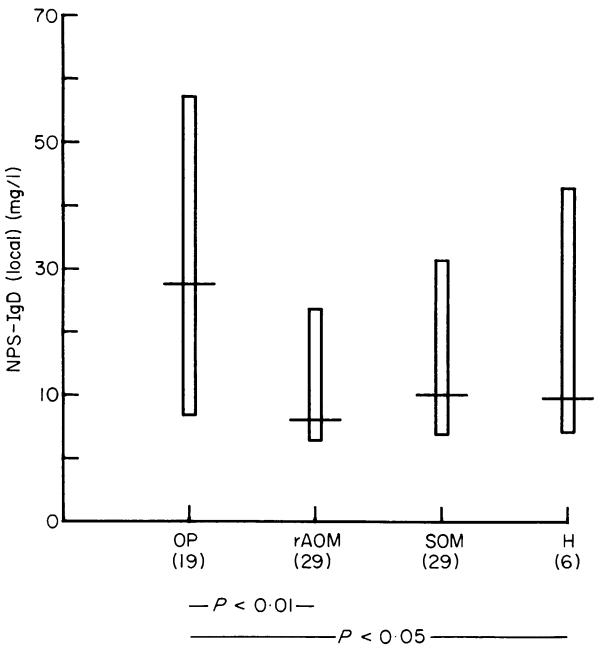

Fig. 4. Levels of locally-produced IgD in nasopharyngeal secretions (NPS-IgD (local)) in otitis-prone children (OP), children with recurrent acute otitis media (rAOM), secretory otitis media (SOM), and healthy children (H). Median levels (horizontal lines) and the interquartile ranges (bars) are indicated. Number of children are given in parentheses. The Kruskal–Wallis test ($P < 0·05$) and the Mann–Whitney U-test ($P < 0·01$) were employed.

Table 1 and Fig. 3. Low quantities of IgD are present at birth, but increasing concentrations were measured already during the first 24 months of age (Fig. 3). However, a steady increase of plasma IgD during childhood could not be demonstrated (Spearman rho = 0·13, $P > 0·1$), and maximum levels of plasma

Table 1. Levels of IgD in cord sera, plasma samples from otitis-prone children (OP), children with recurrent acute otitis media (rAOM), secretory otitis media (SOM), and in healthy children (H), whole-salivary samples, and nasopharyngeal secretions (NPS)

	IgD (mg/l)	(Iqr)	
Cord sera ($n = 44$)	0·098	(0·057–0·122)	
Plasma			
OP ($n = 42$)	11·8	(5·3–30·8)	
rAOM ($n = 50$)	9·3	(3·4–27·4)	
SOM ($n = 51$)	13·2	(2·3–23·6)	$P > 0·40$*
H ($n = 37$)	8·5	(2·4–22·2)	
Whole-salivary ($n = 119$)	< 0·015	(< 0·015–0·098)	
NPS ($n = 83$)			
Total	11·5	(3·3–31·2)	
Local†	10·0	(2·9–31·0)	
Local/total‡	88%	(81–92)	

NPS-IgD are expressed as total and locally produced concentrations. Median levels and interquartile ranges (Iqr) are given.

*Kruskal–Wallis one-way analysis of variance.

†NPS-IgD (total) − plasma-IgD × (NPS-albumin/plasma-albumin).

‡NPS-IgD (local)/NPS-IgD (total) × 100%.

IgD were observed between 48 and 72 months of age. An even distribution of plasma IgD was found among the four groups of children ($P > 0·4$, Table 1). Levels of IgD in NPS (NPS-IgD) and whole-salivary samples are shown in Table 1. Estimation of locally-produced NPS-IgD (NPS-IgD (local)), using the formulas shown in Table 1, revealed that 88% (median) of NPS-IgD was locally produced. Nevertheless, a significant correlation was calculated between plasma-IgD and NPS-IgD (local) (Spearman rho = 0·66; $P < 0·001$). The distribution of NPS-IgD (local) was uneven, as shown in Fig. 4, and significantly higher levels of NPS-IgD (local) were found in OP children than in the other children ($P < 0·05$). In whole-salivary, the IgD content was on the average less than 1:800 of NPS-IgD (Table 1).

Owing to an uneven distribution of IgD-cells in the subepithelial layers of NPT (Figs 1 and 2a), it was necessary to evaluate 30 fields per tissue section to obtain a reliable estimate of the median IgD cell density (Fig. 2a), in line with observations published by Korsrud & Brandtzaeg (1980b). However, as shown in Fig. 2b, the irregular distribution of IgD cells in the individual NPT did not reveal any systematic inferior or superior accumulation of positive cells. In some NPT, practically no positive IgD-cells were found, irrespective of the location of the tissue sections (NPT numbers 1, 5, 6, and 9, Fig. 2b). However, the variation of the IgD cell density throughout the tissue, expressed by the interquartile ranges (Fig. 2b), was found to be proportional to the level of the median cell density, and a statistically significant correlation was observed between these two parameters (Spearman rho = 0·97, $P < 0·01$). Essentially for this reason, we applied the semi-quantitative counting system to the clinical material. In 24 NPT and paired NPS a statistically significant correlation was calculated between the density of IgD cells and levels of NPS-IgD (local) (Fig. 5,

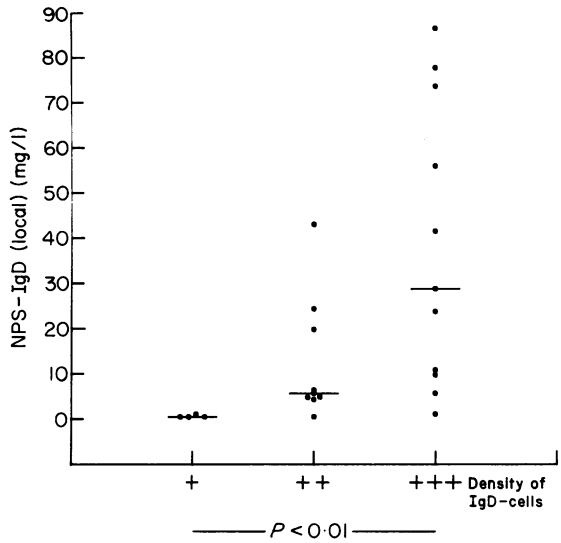

Fig. 5. Levels of locally-produced IgD in nasopharyngeal secretions (NPS-IgD (local)) as a function of the IgD-cell density in nasopharyngeal tonsils from 24 children with varying degrees of otitis-proneness. The IgD-cell density was evaluated semi-quantitatively (see Materials and Methods). Horizontal lines indicate median levels. The Kruskal–Wallis test ($P < 0{\cdot}01$) was employed.

$P < 0{\cdot}01$). Moreover, results from quantification of IgD in paired samples of NPS and MEE showed a statistically significant correlation (Spearman rho = 0·72; $P < 0{\cdot}001$).

DISCUSSION

The placental transfer of IgD is negligible and low quantities are found in cord sera (Table 1). A steady increase of plasma IgD during infancy and childhood has been reported (Johansson & Berg, 1967), but according to our results no correlation of plasma IgD with age could be calculated (Fig. 3). Maximum levels of plasma IgD were observed in children 4–6 years old, corresponding to eight times the mean level previously reported in adults (Dunette *et al.*, 1977).

The idea of a common mucosal immune system has gained support from experimental and clinical studies (review by Scicchitano, Ernst & Bienenstock, 1987). However, results from other studies in humans indicate that the secretory immune system is heterogenous as regards the distribution of mature B cells to the intestinal and upper respiratory tract mucosae (Brandtzaeg *et al.*, 1979; Kett *et al.*, 1986). In this context, the discrepant occurrence of IgD cells, expressed by the relative enrichment of such cells in nasal mucosa, palatine and nasopharyngeal tonsils, is noteworthy (Rowe, Crabbe & Turner, 1968; Brandtzaeg *et al.*, 1979; Korsrud & Brandtzaeg, 1980b). In the present study we found almost equal levels of plasma-IgD and NPS-IgD, but nearly 90% of NPS-IgD proved to be of local origin (Table 1). Nevertheless, we calculated a relatively close correlation between plasma-IgD and NPS-IgD (local). A simple explanation may be that a major part of circulating IgD, especially in cases with raised levels, is of mucosal origin, as also proposed by others (Brandtzaeg *et al.*, 1987). Previously, we observed increased plasma levels of secretory IgA (SIgA) in children with recurrent inflammatory events at the nasopharyngeal level (Sørensen & Nielsen, 1988), which also supports the idea of re-absorption of Igs produced at the mucosal surfaces. Only trace amounts of IgD were found in unstimulated whole-salivary of age-matched children. The IgD levels were generally lower than those reported by Plebani *et al.* (1983), but differences in the age distribution probably explain this discrepancy. Previously, we have reported on levels of SIgA in NPS and unstimulated whole-salivary in young children (Sørensen, 1982). The ratio of NPS-SIgA to salivary-SIgA was on the average 18:1. In contrast the corresponding ratio of IgD was 800:1 (Table 1), indicating a lack of local production of IgD in the oral cavity.

The association between levels of NPS-IgD (local) and the density of NPT-IgD-cells (Fig. 5) indicates that the surface of this lymphoepithelial tissue also functions as an important source of NPS-IgD. However, whether these cells primarily originate from ajoining lymphoid follicles (mantle zone?) or from other and more distant parts of the mucosa-associated lymphoid tissue is as yet unknown, but a question of immediate interest to be solved. Moreover, we found significantly higher levels of NPS-IgD (local) in OP children than in children with a smaller frequency of upper respiratory tract infections (Fig. 4). This observation accords with the findings in palatine tonsils from patients with recurrent tonsillitis (Surjan, Brandtzaeg & Berdal, 1978). Most IgD cells from the extrafollicular area of NPT have previously proved to be J-chain-positive, but incorporation of this polypeptide into the cytoplasmic Ig has never been observed (Korsrud & Brandtzaeg, 1980b). Accordingly, IgD cannot bind the secretory component (SC) and thereby act as a secretory Ig (Brandtzaeg, 1983a, b). This was indirectly confirmed in this study by lack of SC reactivity in NPS-IgD molecules, as previously also shown in MEE-IgD (Sørensen, 1983). During differentiation and maturation of B cell memory clones, rearrangement of the Ig constant heavy-chain genes results in isotype switching of the cell (Rabbitts, Foster & Milstein, 1981; Kuritani & Cooper, 1982). Moreover, results from animal studies indicate that phenotypic switching without the help of T cells may occur under constant exposure to environmental antigens (Cebra *et al.*, 1982). Thus, the cytoplasmic-positive IgD-cells, as found in the NPT, may represent B cell clones in an early phase of differentiation (Brandtzaeg *et al.*, 1979), which for yet unknown reasons, terminate the differentiation process and mature 'too early'. The nasopharynx and the middle ear cavity of OP children are frequently colonized with a variety of microorganisms (Long *et al.*, 1983). It appears likely, therefore, that the recurrent heavy load of microbial antigens influence the immunoregulation of local B cells of early differentiation. The correlation calculated between NPS-IgD and MEE-IgD supports this assumption. Inasmuch as the role of IgD in the secretions is unknown, it is impossible at present to judge the effect of the apparently pathophysiological termination of B cells, as found in the nasopharynx of OP children.

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