Secretor status and humoral immune responses to *Neisseria lactamica* and *Neisseria meningitidis*

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SUMMARY

Non-secretors of ABO blood group antigens are over-represented among patients with meningococcal diseases. Lower levels of secretory IgA reported for non-secretors have been suggested to compromise mucosal defences. Total serum and salivary IgG, IgA and IgM and levels of these isotypes specific for *Neisseria lactamica* and five isolates of meningococci were determined by ELISA for 357 pupils and staff of a secondary school in which an outbreak of meningitis occurred. There were no differences in total or specific levels of serum IgG, IgA or IgM or salivary IgG or IgA of secretors compared with non-secretors. Non-secretors had significantly lower levels of salivary IgM ($P = 0.022$). A similar pattern was observed for levels of IgM specific for *N. lactamica* and five meningococcal isolates. The significance of these results is discussed with reference to the role of secretory IgM in protection of mucosal surfaces in infants.

INTRODUCTION

The ability of the host to resist infection or colonization by microorganisms is partly dependent on the presence of a fully functional mucosal immune system. Although IgA is the most abundant immunoglobulin in exocrine secretions such as tears, saliva and milk, IgG and IgM are also found in these fluids [1, 2]. IgM can also function as a true secretory immunoglobulin [3], reaching the mucosal surface by the identical secretory-component mediated transcellular pathway that transports IgA. The full protective potential of secretory IgA is not present in the infant at birth [4, 5]. Adult levels of secretory IgA are not reached until after 1 year of age [6, 7]. It has been suggested that in infants the presence of IgM compensates partially for the low levels of IgA [7]. IgM to poliovirus and *Escherichia coli* has been found in infants [7, 8].

Protective immunity to disease due to *Neisseria meningitidis* is associated with the presence of an intact complement system and opsonizing or bactericidal antibodies specific for the invading strain [9–11]. These antibodies can be formed through nasopharyngeal carriage of meningococci [12]. The majority of individuals

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who develop invasive disease lack protective antibody to the pathogen [9, 10]. The age range in which meningococcal infection is most prevalent (6 months to 4·5 years) reflects the natural development of antibodies to these pathogens.

Non-secretors of ABO blood group antigens are over-represented among patients with disease due to *N. meningitidis* [13, 14] and among carriers of this bacterium [15]. The lower immunoglobulin levels found in non-secretors compared with secretors have been used to explain the increased susceptibility of non-secretors to rheumatic fever and rheumatic heart disease [16, 17]. In later studies, non-secretors were reported to have lower levels of both serum [18] and salivary [19] IgA. It was suggested that specific immune responses at the mucosal surfaces of non-secretors might be compromised compared with that of secretors. The aims of the present study were to determine:

- (1) if there is a difference in the amount of total serum and salivary IgA, IgG and IgM between secretors and non-secretors;
- (2) if there is a difference in the levels of isotypes specific for *N. lactamica* and *N. meningitidis* in serum or saliva of secretors and non-secretors;
- (3) if there is a correlation between levels of specific anti-meningococcal antibodies in secretions and in serum;
- (4) if carriage of meningococci affects the levels of antibody to these bacteria found in secretors and non-secretors.

MATERIALS AND METHODS

Subjects

Sera and whole saliva specimens (357) were obtained from staff and pupils (most within the 12–18 years age group) of a school in which there was an outbreak of meningococcal disease due to a serogroup B, serotype 4, subtype P1.15 sulphonamide resistant strain (B:4:P1.15). Information regarding carriage of meningococci, secretor status, ABO and Lewis blood group antigens were reported in a previous study [15]. Informed consent to participate in the survey was obtained from parents or guardians of the children.

Isolates of neisseria

An isolate of *N. lactamica* and five different isolates of *N. meningitidis* expressing different serogroup, serotype and subtype antigens were obtained from the freeze-dried strains in the collection of the Infection and Immunity Laboratory, Medical Microbiology Department, University of Edinburgh. The outbreak strain and a B:15:P1.16 sulphonamide resistant isolate were obtained from Dr R. J. Fallon, Meningococcus Reference Laboratory (Scotland), Ruchill Hospital, Glasgow (Table 1). The bacteria were grown on Modified New York City (MNYC) agar [20] for 24 h in a humidified atmosphere with 10% CO₂.

Determination of total immunoglobulin isotype levels

The total amount of IgM and IgA were assayed by a capture ELISA method. IgG from serum and saliva was coated directly onto ELISA plates. For the measurement of total IgM and IgA, the wells of polystyrene microtitre plates were coated overnight at 4 °C with either 100 µl of mouse monoclonal anti-human IgM

Table 1. *Bacterial isolates*

Strain	Source	Serogroup	Serotype	Subtype
<i>N. meningitidis</i>				
A11	Patient	B	15	P1.16
A43	Patient	B	4	P1.15
A41	Carrier	B	4	—
A26	Carrier	C	4	—
A48	Carrier	NG*	4	—
<i>N. lactamica</i>				
L01	Carrier	NG	—	—

* Non-groupable.

Clone No. MB-11, lot 69F-4807 (1/500) (Sigma, Poole, Dorset, UK) or mouse monoclonal anti-serum IgA Clone No. GA-112, lot 99F-4803 (1/500) (Sigma) diluted in coating buffer (15 mM NaCO₃, 35 mM NaH₂CO₃, 3 mM NaN₃; pH 9.6). The coated plates were washed three times with phosphate buffered saline (PBS) (0.16 M-NaCl, 8 mM-NaP₂HPO₄, 1 mM-KH₂PO₄, 3 mM-KCl; pH 7.2) containing 0.01 % bovine serum albumin (BSA) and Tween 20 (0.05 % v/v) (PBS-Tween) and blocked with BSA (1 %) in PBS (blocking buffer) for 30 min. After washing, 50 µl of serum (1/2000) or saliva (1/10) diluted in blocking buffer were added and incubated for 2 h at room temperature. Plates for detecting IgM were washed and 50 µl of sheep µ-chain-specific anti-human IgM (1/80) (Scottish Antibody Production Unit, Lanarkshire, Scotland) (SAPU) were added. After washing, 50 µl of horseradish peroxidase conjugated (HRP) donkey anti-sheep/goat IgG (1/20) were added to the plates for 1 h. For IgA plates, 50 µl HRP rabbit anti-human µ-chain-specific IgA (1/500) (Dako, High Wycombe, Bucks, UK) were added to the plates and incubated for 2 h.

Finally, the plates were washed and 50 µl of phosphate citrate buffer (0.1 M-NaHPO₄, 0.1 M citric acid) containing the substrate *O*-phenylene-diamine (0.4 mg/ml, pH 5.0) activated by 0.02 % H₂O₂ (30 % v/v). The colour was allowed to develop in the dark and the reaction was stopped after 20–30 min by adding 50 µl of H₂SO₄ (12.5 %).

To determine total IgG, 100 µl of serum (1/50000) or saliva (1/2) diluted in coating buffer were added to the microtitre plates and the plates incubated overnight under the conditions used, none of the reagents was at a limiting concentration. After washing, 50 µl of HRP sheep anti-human IgG (1/20) (SAPU) diluted in blocking buffer were added for 2 h. The plates were then treated as for IgA and IgM.

Optical density (OD) at 490 nm was determined by an ELISA plate reader (Dynatech) and corrected by subtracting the OD of the corresponding blank. Samples were tested in duplicate and the readings averaged. ELISA readings were converted to mg/ml by extrapolation from the curve that was constructed from readings obtained with standard human serum: IgG = 1140 mg/dl; IgA = 250 mg/dl; IgM = 114 mg/dl (Behring lot no. 041024). A series of twofold dilutions of standard human serum (Behring, London, UK) containing known concentrations of immunoglobulin (mg/ml) were tested with the specimens from the children.

Whole cell enzyme linked immunosorbent assay

Sera and saliva were examined for anti-meningococcal antibodies by a whole bacterium ELISA. *N. meningitidis* and *N. lactamica* were cultured overnight on MNYC agar at 37 °C. Large batches of microtitre plates were coated with bacteria to minimize variations due to different antigen preparations and coating procedures. Plates were coated overnight at 4 °C with 100 µl of one of the six bacterial isolates (6.6×10^7 bacteria/ml). The plates were washed and blocked with PBS containing BSA (1%). The buffer was removed and plates washed with washing buffer. Undiluted serum or saliva (50 µl) was added to the wells and incubated at room temperature for 2 h. The assay was continued in the same way as ELISA for total antibodies. The assay for total and specific antibodies were determined at the same time under the same conditions.

Statistical methods

The statistical analysis of the data was performed with the package SPSS/PC+. The results were summarized by geometric means, since the logarithmic values were more normally distributed than the raw data. The significance levels for differences between groups were examined with the Mann-Whitney *U* test and a *P* value of < 0.05 was regarded as significant. The association between levels of antibodies in serum and in saliva was assessed by Spearman rank correlation.

RESULTS

Total IgA, IgG and IgM antibodies of secretors and non-secretors

The specificity of the ELISA for detection of IgM, IgG and IgA was assayed with purified IgM, IgG and IgA (data not shown). There was no cross reaction between IgA, IgG and IgM. There was no marked difference in total immunoglobulin levels between sera from secretors and non-secretors. Non-secretors had higher levels of serum IgM antibody compared with secretors, but the difference was not statistically significant. There was no difference in the levels of IgA or IgG in the saliva from secretors compared with non-secretors; but, there was significantly more total IgM in the saliva of secretors ($P = 0.0274$) (Table 2).

There was no difference in serum or salivary immunoglobulin levels of smokers (37) compared with non-smokers (320).

Specific immune responses to Neisseria species

There was no marked difference in antibody levels to the isolates in sera of secretors compared with non-secretors, except for IgM to the non-groupable serotype 4 isolate (Table 2).

There were significant differences in the mean specific salivary IgM immunoglobulin levels of secretors and non-secretors. Non-secretors had significantly lower levels IgM for *N. lactamica* and each of the meningococcal isolates tested (Table 2). Statistical comparison of IgA and IgG antibody levels in saliva was also performed, but there was no difference between secretors and non-secretors. For both secretors and non-secretors, the highest levels of IgM were observed for the NG:4 isolate.

Table 2. Geometric mean levels of total and specific IgM of secretors and non-secretors

	Non-secretors	Secretors	P
Serum	(n = 107)	(n = 171)	
Total IgM*	1.27	1.08	0.05
Specific			
<i>N. lactamica</i>	9.7	7.7	0.12
B:15:P1.16	10.5	8.5	0.15
C:4	6.0	5.3	0.25
B:4	22.4	14.7	0.11
NG:4	26.8	22.2	0.03
B:4:P1.15	5.2	4.8	0.48
Saliva	(n = 129)	(n = 208)	
Total IgM	63.0	87.0	0.027
Specific			
<i>N. lactamica</i>	1.8	2.5	0.0000
B:15:P1.16	2.1	3.1	0.0000
C:4	2.9	3.6	0.040
B:4	2.2	3.5	0.0000
NG:4	4.7	5.9	0.017
B:4:P1.15	1.9	2.9	0.0008

* Unit for mean total serum IgM levels are expressed in mg/ml; unit for mean total and specific salivary IgM levels and specific serum IgM levels expressed in $\mu\text{g/ml}$.

Table 3. Geometric mean of immunoglobulin levels of carriers and non-carriers

		Mean immunoglobulin levels ($\mu\text{g/ml}$)		P
		Carriers (n)	Non-carriers (n)	
Serum				
Isotype strain				
IgA	C:4	18.0 (67)	13.0 (209)	0.03
IgA	B:4:P1.15	9.5 (66)	8.4 (207)	0.02
IgM	C:4	12.4 (68)	4.1 (210)	0.0000
IgM	B:4:P1.15	8.3 (66)	6.5 (207)	0.0025
IgG*	C:4	5.3 (68)	4.2 (205)	0.01
IgG	B:4:P1.15	7.2 (64)	6.1 (207)	0.0033
Saliva				
IgA	NG:4	9.7 (89)	6.6 (243)	0.01
IgM	NG:4	6.5 (89)	5.1 (243)	0.03

* Unit for mean total serum IgG are expressed in mg/ml.

The correlation between serum and salivary antibodies

Analysis by Spearman correlation test found no correlation between the levels of IgG, IgA and IgM antibodies in serum and saliva ($P > 0.05$). This suggests that the increased levels of secretory IgM found among secretors are due to locally produced immunoglobulins.

Carriage of meningococci and immunoglobulin levels

Compared with non-carriers, carriers of meningococci had significantly higher levels of salivary IgA and IgM to a NG:4 strain and serum antibodies to the

outbreak strain and to a C:4:- isolate (Table 3); however, analysis with respect to both secretor status and carriage revealed that immunoglobulin levels still differed according to secretor status when carriage was taken into account.

There was no difference in salivary immunoglobulins to the outbreak strain; but there were significantly higher levels of IgA and IgM to the NG:4 isolate.

DISCUSSION

An association between non-secretion and meningococcal disease has been reported in studies of patients in Scotland, Iceland and Nigeria [13, 14]. In this study we tested the hypothesis that there might be differences in the humoral immune responses of secretors and non-secretors that could contribute to the apparent increased susceptibility of non-secretors to meningococcal disease. Both serum and salivary immunoglobulin levels were examined.

Conflicting results have been reported for the differences in immunoglobulin levels between secretors and non-secretors; however, most of these studies measured total amounts of immunoglobulin in serum and saliva [18, 19, 21]. Lower levels of both serum [19] and salivary IgA [18] were reported for non-secretors, suggesting that specific immune responses at the mucosal surfaces of non-secretors might be reduced compared with secretors. Blackwell and her colleagues [21] did not confirm the earlier observation with single radial immunodiffusion; higher levels of IgA were associated with the presence of meningococci in the individuals from whom the saliva was obtained. There was no difference in the mean levels of total IgA in the saliva of secretors compared with non-secretors from whom no *Neisseria* spp. were isolated.

There was no difference between secretors and non-secretors in total or specific levels of salivary IgA or IgG; however, non-secretors had significantly less total IgM in their saliva compared with secretors. These differences were also observed for salivary IgM to *N. lactamica* and to five isolates of meningococci expressing different combinations of serogroup, serotype and subtype antigens. There was no correlation between levels of serum IgM and secretory IgM in saliva, suggesting that salivary IgM is locally produced and has not leaked from the serum. Although serum and saliva of carriers had significantly higher levels of antibodies to some of the neisseria isolates, the effect of secretor status on IgM remained after adjustment for the effect of carriage.

A major biological role of high molecular weight, polyvalent IgA and IgM secretory antibodies might be to provide a first line of defence against particulate and polyvalent antigens such as bacteria. Individuals with IgM deficiency appear at risk of disseminated meningococcal disease [22, 23]. It has been demonstrated that locally produced IgM in IgA-deficient patients exhibits anti-virus activity [24].

The lower levels of secretory IgM in non-secretors might contribute to susceptibility to colonization, particularly among infants under the age of 12 months in whom secretory IgM is the major class of antibody on mucosal surfaces. The presence of secretory IgM in early infancy has been suggested to compensate for the absence of secretory IgA [7]. IgM had been detected in saliva of infants who were as young as one month of age [25]. If secretory IgM provides a crucial host

defence during this period of life the lower levels of secretory IgM found for non-secretors might contribute to their apparent susceptibility to meningococcal disease. This hypothesis is under investigation at present.

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