Salivary IgA from the sublingual compartment as a novel noninvasive proxy for intestinal immune induction

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Whole-saliva IgA appears like an attractive noninvasive readout for intestinal immune induction after enteric infection or vaccination, but has failed to show consistent correlation with established invasive markers and IgA in feces or intestinal lavage. For reference, we measured antibodies in samples from 30 healthy volunteers who were orally infected with wild-type enterotoxigenic *Escherichia coli*. The response against these bacteria in serum, lavage, and lymphocyte supernatants (antibody-in-lymphocyte-supernatant, ALS) was compared with that in targeted parotid and sublingual/submandibular secretions. Strong correlation occurred between IgA antibody levels against the challenge bacteria in sublingual/submandibular secretions and in lavage ($r = 0.69$, $P < 0.0001$) and ALS ($r = 0.70$, $P < 0.0001$). In sublingual/submandibular secretions, 93% responded with more than a twofold increase in IgA antibodies against the challenge strain, whereas the corresponding response in parotid secretions was only 67% ($P = 0.039$). With $>$ twofold ALS as a reference, the sensitivity of a $>$ twofold response for IgA in sublingual/submandibular secretion was 96%, whereas it was only 67% in the parotid fluid. To exclude that flow rate variations influenced the results, we used albumin as a marker. Our data suggested that IgA in sublingual/submandibular secretions, rather than whole saliva with its variable content of parotid fluid, is a preferential noninvasive proxy for intestinal immune induction.

INTRODUCTION

The mucosal immune system is characterized by epithelial export of locally produced polymers (mainly dimers) of immunoglobulin A (IgA) released to the lumen as secretory IgA (SIgA) antibodies.¹ It depends on an integrated network of immune cells migrating from inductive mucosa-associated lymphoid tissue to exocrine effector sites, particularly the gut lamina propria, airways, and salivary glands. Mucosal B cells are activated in regional mucosa-associated lymphoid tissue structures such as gut-associated lymphoid tissue (GALT) dominated by Peyer's patches in the distal ileum; after class switching to IgA expression in GALT, the memory/effector

cells migrate to draining lymph nodes, such as the mesenteric lymph nodes, and further on to peripheral blood.² These IgA + cells bear homing receptors (adhesion molecules and chemokine receptors) that direct their extravasation in regional and distant mucosal effector sites where they undergo final differentiation to polymeric IgA-producing plasmablasts and plasma cells. Consequently, because of clonal expansion and compartmentalized homing of GALT-derived B cells, identical SIgA antibody specificities are dispersed along the intestine and also to distant mucosal effector sites.^{3–5} The leukocyte integrin $\alpha 4\beta 7$ assists this homing by its binding to the ligand MAdCAM-1, which is mainly expressed by the intestinal

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vascular endothelium.³ In addition, the chemokine receptor CCR10 is a general mucosal homing molecule directing B cells to exocrine sites where there is epithelial production of its ligand CCL28.³ Several studies in both mice and humans have suggested that intestinal immune induction may become disseminated from GALT to the salivary glands.^{6–8} However, activated B cells expressing CCR10 may also be derived from human nasopharynx-associated lymphoid tissue such as the adenoids and palatine tonsils.⁹

Both enzyme-linked immunospot and antibody-in-lymphocyte-supernatant (ALS) assays are reported to be well correlated with intestinal immune responses when the circulating B cells are collected in a narrow time window (approximately 7 days) after intestinal immune stimulation.^{10,11} Although ALS is less laborious than enzyme-linked immunospot, both methods require blood samples and advanced laboratory methods including sterile working procedures, cell separation, cultivation facilities, and incubators. Accordingly, there is a need to develop a noninvasive, simple readout method to evaluate intestinal immune induction in field trials of enteric vaccines. If it could be demonstrated that IgA antibodies in saliva indeed reflect consistently immune responses in the gut, salivary secretion might be useful as a proxy to evaluate intestinal immunity. Compared with intestinal lavage and fecal specimens, saliva is easily collected, and salivary IgA is less prone to proteolytic degradation than IgA in intestinal secretions. Nevertheless, several studies using whole saliva have failed to show a convincing relationship between salivary IgA antibodies and direct readouts for intestinal immunity such as antibodies in feces.^{12–15} Leakage of antibodies from plasma was also encountered, as discussed later.¹⁶ Svennerholm's group has worked extensively on this methodological problem for several years, and in their latest technological presentation,¹⁷ they concluded that "Antibody responses in saliva poorly reflect intestinal responses". A similar statement was made in their review from 2012.¹⁸ However, salivary IgA in targeted samples from the major salivary glands has to our knowledge not previously been tested for such a relationship.

Our original hypothesis was that the production of IgA antibodies in sublingual/submandibular glands reflects immune responses in the gut more consistently than comparable IgA production in parotid glands. One study in rats showed differential homing of both T and B cells to the submandibular vs. the parotid gland.¹⁹ Nevertheless, our idea was based on a review of the literature related to untreated celiac disease or gluten-sensitive enteropathy.²⁰ These patients have elevated levels of IgA antibodies against gluten, both in intestinal secretions and serum.²¹ As a sign of gluten-induced GALT activation, they also have circulating IgA-producing gluten-specific plasmablasts, which can be detected by enzyme-linked immunospot assay.²² It could therefore be anticipated that IgA antibodies to gluten would occur in saliva. In the 1990s, this possibility was explored in a search of a noninvasive approach to screen for celiac disease. In two studies of unstimulated whole saliva, the discrimination of confirmed celiac patients

was quite satisfactory,²³ or even better than serum IgA antibodies.²⁴ Stimulated parotid secretion, however, showed only little²⁵ or virtually no²⁶ elevation of IgA antibodies to gluten. These results suggested preferential B-cell homing from GALT to the sublingual/submandibular glands.

To test this theory, we analyzed various body fluids sampled during an experimental study where volunteers were infected orally with a wild-type enterotoxigenic *Escherichia coli* (ETEC) strain. Specifically, targeted sampling of unstimulated sublingual/submandibular and parotid secretions was carried out with oral swabs at different time points after infection and analyzed for antibodies against the challenge ETEC strain and a heterologous ETEC strain. The IgA antibody levels in the salivary secretions were compared with the corresponding IgA responses observed in intestinal lavage and in ALS.

RESULTS

Study population

A total of 30 healthy young adult volunteers were infected with the wild-type ETEC strain TW10598.²⁷ Peripheral venous blood, salivary secretions, intestinal lavage, and lymphocytes for ALS were collected before infection (day 0) and at different time points thereafter.

Antibody response to ETEC in serum

Antibodies directed against the challenge strain TW10598 and a heterologous ETEC strain (H10407) were measured in arbitrary units (AU) by flow cytometry with live bacteria as the target. The serum IgA response was reported in our initial publication describing the clinical setting of the volunteers participating in the study.²⁷ In the pre-infection sera (day 0), the IgG geometric mean (GM) level of antibodies directed against TW10598 was 37.9 AU per ml. The highest GM serum IgG level was observed at day 10, with a titer of 425.1 AU per ml. As for serum IgA, there was a strong serum IgG anti-TW10598 response from day 0 to days 7, 10, and 28 ($P < 0.0001$ to all time points) (Figure 1). The fold increase in GM IgG antibody levels were 5.0 (confidence interval, CI: 3.1, 8.1), 11.2 (CI: 6.7, 18.9),

Figure 1 Serum IgG antibody levels against the TW10598 ETEC challenge strain. Antibodies against live bacteria were measured as AUs by flow cytometry in 30 adult volunteers at different time points, pre- (day 0) and post-experimental infection. Circles represent the IgG antibody level of each volunteer; horizontal lines indicate geometric means and 95% confidence intervals. AUs, arbitrary units; ETEC, enterotoxigenic *Escherichia coli*; Ig, immunoglobulin.

and 10.1 (CI: 6.4, 15.9). Similarly to the IgA response,²⁷ there was no detectable serum IgG response against the heterologous ETEC strain (H10407) (GM titers of 9.0, 9.1, 8.9, and 9.6 AU per ml at days 0, 7, 10, and 28, respectively).

IgA response to ETEC in intestinal lavage and ALS

Lavage fluid from 29 and ALS from all 30 participants were available for analysis. IgA antibodies against the ETEC challenge strain were measured by an established flow-cytometric method with live bacteria.²⁸ When testing dilutions of intestinal lavage with the TW10598 strain against an antibody to bound secretory component (SC), low SIgA titers were detected in the pre-challenge (day 0) lavage (**Figure 2a**), with a GM titer of 10.2 AU per ml (CI: 9.3, 11.3). On day 10 of infection, however, the level of specific SIgA had increased to 78.6 AU per ml (CI: 54.7, 112.7). Ninety percent (26 of 29) of the volunteers experienced a greater than twofold increase. The increase of specific SIgA in lavage was pronounced also after normalization of the antibody level against total SIgA (**Figure 2b**).

There was no detectable IgA response against the challenge strain in ALS collected prior to infection. On day 7, specific IgA was observed in 26 of the 30 samples with a GM IgA level of 5.6 AU per ml (CI: 3.4, 9.2) (**Figure 2c**). There was a strong correlation between SIgA antibody levels in intestinal lavage and IgA antibody levels in ALS ($r=0.84$, $P<0.0001$).

Three lavage samples revealed weak IgA reactivity against the heterologous ETEC strain H10407 at day 10; the same samples showed a strong IgA response against the challenge strain (**Figures 3a, b**). There was no response against strain H10407 in any of the ALS preparations (**Figure 3c**), attesting to the specificity of the assay.

IgA, SIgA, and IgA subclass responses to ETEC in salivary secretions

The levels of IgA antibodies against the two live ETEC strains were measured in sublingual/submandibular and parotid secretions by flow cytometry. Owing to incomplete registration of the collected sample volumes, sublingual/submandibular secretions were unavailable for three subjects at day 0, one subject at day 7, and one subject at day 28; such information was

also lacking for parotid samples from the same three participants at day 0, and for one of these, none of the post-infection samples were correctly collected.

From the day of challenge to 10 days thereafter, we observed a substantial increase in IgA directed against the challenge strain TW10598, both in sublingual/submandibular and parotid secretions. This was evident even at day 7, with a peak at day 10 and a subsequent decline at day 28 (**Figures 4a, b**). The proportion of individuals responding with greater than a twofold increase in anti-TW10598 titers was 93% (25/27) when assessed in the sublingual/submandibular secretions vs. 67% (18/27) when assessed in the parotid secretions (Exact McNemar's test for paired samples, $P=0.039$) (**Figures 4c, d**). In the pre-challenge sublingual/submandibular secretions, the IgA antibody GM titer was 0.62 AU per ml (CI: 0.4, 1.1). At day 10, it had risen to 5.8 AU per ml (CI: 3.5, 9.4), corresponding to a 10.4-fold (CI: 5.8, 18.8) increase (**Figure 4a**). In the pre-challenge parotid secretions, the GM IgA antibody titer against strain TW10598 was 0.56 AU per ml (CI: 0.3, 1.1); at day 10, it was 4.7 AU per ml (CI: 2.1, 10.3), corresponding to a 9.4-fold (CI: 4.2, 21.0) increase (**Figure 4b**). When a random subset of 16 parotid samples was tested against the heterologous ETEC strain (H10407), no IgA response was revealed from pre-challenge to 10 days post challenge, in contrast to the highly significant response against the challenge strain TW10598 (**Figure 4e**).

Samples from day 0 and day 10 were also analyzed with an antibody against bound SC of SIgA. There was a convincing SIgA response against the challenge strain in both sublingual/submandibular and parotid secretions. A strong correlation was observed between SIgA levels in intestinal lavage and specific SIgA levels in sublingual/submandibular secretions ($r=0.69$, $P<0.0001$) when samples from day 0 and day 10 were analyzed, and also between specific IgA in ALS (samples at day 0 and day 7) and specific SIgA in sublingual/submandibular secretions ($r=0.70$, $P<0.0001$).

There was no detectable IgG activity against the challenge strain in sublingual/submandibular or parotid secretions, attesting to the assumption that the analyzed IgA antibodies in these specimens were derived from local plasma cells rather than by leakage from peripheral blood.

Figure 2 Induction of IgA antibodies in intestinal lavage fluid and of ALS after oral challenge with ETEC bacteria was quantified against live ETEC bacteria by flow cytometry. (a) Intestinal infection with the ETEC challenge strain TW10598 induced specific SIgA responses in lavage fluid ($N=29$). (b) This response was convincing also after normalization of the SIgA levels against total SIgA in lavage. (c) Intestinal infection similarly induced a strong IgA antibody response in ALS ($N=30$). ALS, antibodies in lymphocyte supernatant; AU, arbitrary unit; ETEC, enterotoxigenic *Escherichia coli*; Ig, immunoglobulin; SIgA, secretory IgA.

Figure 3 Comparison of IgA responses in intestinal lavage fluid, ALS, and parotid secretion measured against two different ETEC strains. In contrast to the challenge strain (TW10598), there were no significant IgA responses against the heterologous ETEC strain (H10407). (a) Flow cytometry histograms showing the binding profile of lavage fluid from two subjects at day 0 and day 10, against the challenge strain (TW10598, upper panel) and the heterologous strain (H10407, lower panel). The filled histograms reflect day 0 and the open histograms day 10. Both subjects showed high responses against strain TW10598, whereas only subject 25 showed a weak response against strain H10407. (b) Only 3 of 29 subjects revealed a slight IgA response in lavage fluid against H10407 at day 10; these 3 also had a relatively high response against strain TW10598 ($N = 29$). (c) None of the 30 subjects showed any response against strain H10407 in ALS at day 7, which contrasts with the strong response against the challenge strain ($N = 30$). Horizontal lines indicate geometric means and 95% confidence intervals. ALS, antibodies in lymphocyte supernatant; ETEC, enterotoxigenic *Escherichia coli*; GMFI, geometric mean PE fluorescence intensity; Ig, immunoglobulin; PE, phycoerythrin.

To explore whether GALT might be the origin of TW10598-specific sublingual/submandibular plasma cells, the IgA1 and IgA2 subclasses distribution of antibodies in sublingual fluid and intestinal lavage was compared for 16 subjects where paired sample were available (Figure 5). On the average, the ratio was the same, but individual differences suggested that different parts of GALT (distal or proximal) could have been stimulated by the ETEC challenge. In addition, in this assay, the control strain H10407 showed no reactivity ($N = 16$).

To confirm that the elevation of specific salivary IgA levels was a result of increased local production and not caused by a reduced salivary flow rate after infection, we measured the concentration of albumin as an internal surrogate marker in sublingual/submandibular and parotid secretions (the rationale for this approach is explained under Discussion). The GM albumin concentration in sublingual fluid did not increase from day 0 (19.3 mg ml^{-1} (95% CI: 13.8, 26.9)) to day 10 (22.9 mg ml^{-1} (95% CI: 16.3, 32.3)); the corresponding values in parotid fluid were 40.4 mg ml^{-1} (95% CI: 27.4, 59.6) and 56.0 mg ml^{-1} (95% CI: 34.5, 90.9) (Figures 6a, b). For the sublingual/submandibular secretions, the difference between the day 10 and day 0 GM levels was negligible, that is 1.18-fold (95% CI: 0.83, 1.69; $P = 0.34$), while in the parotid secretions the GM increased 1.47-fold (95% CI 1.03, 2.08; $P = 0.034$). Notably, the ratios of specific IgA titers:albumin concentrations ($\mu\text{g ml}^{-1}$) showed a substantial increase from pre-challenge to 10 days after ETEC infection. Thus, the GM of these ratios increased 8.8-fold (95% CI: 4.7, 16.4; $P < 0.0001$) from 32.0 in the sublingual/submandibular secretions, and 6.4-fold (95% CI: 2.8, 14.9; $P = 0.0002$) from 14.0 in the parotid secretions (Figures 6c, d).

Sensitivity and specificity of IgA in targeted salivary secretions as a proxy for gut immune induction

Using >twofold ALS IgA response as the reference, we estimated the sensitivity of the sublingual/submandibular secretions (also >twofold IgA response) and found it to be 96% (24/25), while the corresponding value for intestinal lavage was 92% (23/25) and for parotid secretions, it was only 67% (16/24).

Note also that all our volunteers were infected with ETEC and almost all had a strong ALS response to the challenge strain; our study was therefore not designed to calculate the specificity of salivary IgA titers. However, judging by the strong correlation ($r = 0.70$; < 0.0001) between sublingual SIgA anti-TW10598 and specific IgA in ALS, we have no reason to believe that our salivary assay reflects poor specificity.

In addition, we addressed the issue whether the same individuals were strong responders in sublingual samples but poor responders in parotid samples. We found that 17 individuals responded with >twofold increase of IgA antibodies both in sublingual and parotid secretions, 1 individual had no such «significant» response, in either sublingual or parotid fluid, 8 individuals responded significantly in sublingual but not in parotid fluid, while 1 individual responded «significantly» in parotid but not in sublingual fluid. These results attest to our idea that sublingual fluid is a better sample than parotid fluid (and thereby whole saliva) to reveal IgA induction in the gut.

Total IgA and SIgA levels were raised in salivary secretions and intestinal lavage after ETEC infection

We observed increased total IgA levels in saliva after ETEC challenge both in sublingual and in parotid fluid (Figures 7a, b). The greatest GM elevation occurred in sublingual fluid from day 0 ($260.3 \mu\text{g ml}^{-1}$; 95% CI: 179.3,

Figure 4 Kinetics of salivary IgA responses after oral ETEC challenge. The kinetics of salivary IgA response against the challenge strain in sublingual (a) ($N=27, 29, 30$, and 29 on day 0, 7, 10, and 28, respectively) and parotid (b) ($N=27, 29, 29, 29$) secretions at different time points after infection. There was a substantial increase in the IgA titer following intestinal infection compared with the pre-challenge level, both in sublingual and parotid secretions. At day 10, there were more responders in sublingual secretions (c) than in parotid secretions (d). The solid diagonal line reflects line of identity, whereas the stippled diagonal line represents twofold increase. (e) Sixteen randomly selected parotid fluid samples collected at day 0 and day 10 after challenge were tested for SIgA antibodies against live H10407 and TW10598 strains. AU, arbitrary unit; ETEC, enterotoxigenic *Escherichia coli*; Ig, immunoglobulin; SIgA, secretory IgA.

Figure 5 ETEC-specific IgA subclass responses in intestinal lavage and sublingual fluid. IgA1 and IgA2 antibodies against the live challenge strain were measured at day 10 in paired lavage and sublingual fluid samples by flow cytometry. Horizontal lines indicate geometric means and 95% confidence intervals. Paired samples from 16 individuals were available for these analyses. ETEC, enterotoxigenic *Escherichia coli*; Ig, immunoglobulin.

377.9) to day 10 ($419.7 \mu\text{g ml}^{-1}$; 95% CI: 285.3, 617.2), and in parotid fluid from day 0 ($243.8 \mu\text{g ml}^{-1}$; 95% CI: 141.8, 419.1) to day 7 ($506.8 \mu\text{g ml}^{-1}$; 95% CI: 274.7, 935.0). As there may be some leakage of plasma IgA into the oral cavity, the total salivary SIgA levels in samples collected at day 0 and day 10 were quantified with an antibody against bound SC. There was

a significant GM increase of SIgA from the pre-challenge at day 0 ($555.1 \mu\text{g ml}^{-1}$; 95% CI: 409.4, 752.7) to day 10 ($756.3 \mu\text{g ml}^{-1}$; 95% CI: 556.3, 1028.0) ($P=0.04$) in the sublingual fluid, and a similar trend in parotid secretions from day 0 ($435.7 \mu\text{g ml}^{-1}$; 95% CI: 306.2, 619.9) to day 10 ($621.5 \mu\text{g ml}^{-1}$; 95% CI: 401.4, 962.2) ($P=0.08$).

An even greater increase of total SIgA was seen in intestinal lavage, amounting to almost a doubling from day 0 to day 10 after challenge, i.e., from $667.2 \mu\text{g ml}^{-1}$ (95% CI: 475.1, 936.8) pre-challenge to $1,163 \mu\text{g ml}^{-1}$ (95% CI: 951.8, 1421) 10 days post challenge ($P=0.002$).

DISCUSSION

ALS is considered an excellent readout for gut immune induction,¹¹ but it depends on a volume of blood that cannot easily be obtained from children in vaccine trials. This has inspired us, despite previous studies having failed (see Introduction), to test salivary IgA as a noninvasive proxy for intestinal immunity.

We have documented that experimental intestinal infection with ETEC after oral administration induces sublingual/submandibular and parotid IgA directed against the challenge strain. This is likely to reflect that activated memory/effector B cells home to the salivary glands from the GALT. Such a B-cell migration pathway was suggested many decades ago both for mice and for humans.^{6,7,20} A more recent study of mutations in

Figure 6 The concentration of albumin remained fairly unchanged in sublingual/submandibular (a) and the parotid (b) secretions from day 0 to day 10, thus indicating comparable salivary flow rates at these time points. The horizontal lines show the geometric mean concentrations at each time points. When the specific anti-TW10598 IgA titer was divided by the albumin concentration, the ratio at day 10 was substantially ($P < 0.0001$) higher than at day 0, particular for the sublingual/submandibular secretions, thus attesting to an immune response for IgA. The solid diagonal line reflects the line of identity, whereas the stippled diagonal line represents twofold increase. The nonresponders at and below the diagonal line in panels c and d represent the same specimens as the nonresponders in Figures 3c and d. Ig, immunoglobulin.

the IgV heavy-chain genes of plasma cells from human duodenal mucosa and salivary glands did indeed suggest a common origin from GALT.²⁹

The magnitude of the salivary IgA antibody response was much lower than the corresponding plasma IgA response. However, although the half-life of plasma IgA is around 1 week, locally produced IgA is continuously exported to saliva and flushed away. It is highly unlikely that the salivary IgA antibodies were transudated from plasma as we obtained similar overall responses with SIgA (with a detection antibody against bound SC rather than against the α -chain of SIgA). The specificity of the anti-SC used to quantify SIgA was documented in that it showed no binding to bacteria coated with serum IgA. However, the results for individual volunteers with anti- α -chain and anti-SC could vary both for biological and technical reasons.

The ratio of specific IgA1: IgA2 to the ETEC challenge strain was, on average, the same in intestinal lavage and sublingual fluid. This suggested a common origin for the plasma cells producing the antibodies. However, GALT and mucosa-draining lymph nodes differ in the distal and proximal gut.³⁰ In the upper part of the small bowel, retinoids from bile may exert an important enhancing effect on the immune response via retinoic acid. In a recent study, retinoic acid signaling in B cells was shown to be essential for their IgA response interacting with the microbiota.³¹ This could explain

the relatively pronounced IgA1 titer against the ETEC challenge strain in several test subjects. It is well known that the IgA plasma cell distribution differs among secretory tissues, with a dominance of the IgA2 phenotype only in the distal gut.³²

The fluid secretion rate influences the level of salivary IgA,²⁰ but it was not possible to monitor this variable for the small collected samples. We therefore used the approach proposed by Gleeson and coworkers,^{33,34} namely, to apply the concentration of albumin as an internal surrogate marker for this variability as long as absence of IgG shows no transudation of plasma proteins (e.g., as in the neonatal period). Albumin is not produced locally in the salivary glands, and so theoretically, only the flow rate will influence its level. However, we admit that it is no perfect marker as its transudation may be subjected to uncontrolled events such as slight trauma to the oral mucosa. We were therefore positively surprised to see that the variability of its concentration was modest; on average, it showed no change from pre- to post-ETEC challenge in the sublingual fluid, thus excluding decreased flow rate as an explanation for the observed IgA ETEC response. This result was further confirmed as the ratio between the specific IgA titer and the albumin concentration was substantially higher on day 10 than on day 0. The use of albumin as a marker was further supported by the lack IgG antibodies against the challenge strain in salivary secretions despite high plasma levels of such antibodies.

Figure 7 Total IgA concentrations increased in salivary secretions after intestinal ETEC challenge. A significant increase of IgA concentration was noted both in sublingual (**a**) and parotid (**b**) secretions the first days after intestinal infection as measured by ELISA. Each circle represents the IgA concentration from one individual, the horizontal bars and the error bars indicate the geometric mean and 95% confidence intervals (*N* as indicated in Figure 3). The differences between log-transformed IgA concentrations at different time points were assessed with paired *t*-test. ELISA, enzyme-linked immunosorbent assay; ETEC, enterotoxigenic *Escherichia coli*; Ig, immunoglobulin.

That a few of the volunteers had a high anti-ETEC titer before the experimental challenge is to be expected as they might still be colonized with *E. coli* strains that share epitopes with our challenge strain. Furthermore, some of the volunteers could have travelled to ETEC endemic countries up to 9 months before they were challenged²⁷ and might thus have been infected with ETEC similar to our challenge strain.

The measured antibody response was clearly specific for the ETEC challenge strain. When tested against a heterologous ETEC strain (H10407), IgA binding to the bacteria was observed neither in serum nor in salivary secretions. This could in part be explained by the method we used, i.e., surface binding to live bacteria by flow cytometry which in our hands did not cause nonspecific results as encountered when testing for specific antibodies by enzyme-linked immunosorbent assay. This approach detects antibodies that bind with high affinity to surface-exposed epitopes, whereas internal and concealed antigenic structures remain unavailable. Antibody binding to live bacteria has, to our knowledge, not been used for salivary IgA antibody determination before, and this method mimics the *in vivo* immune interaction that aids bacterial elimination and clears intestinal infection (whereas neutralization

antibodies against secreted toxins may alleviate symptoms and disease).

We measured the serum antibody levels against heat-labile enterotoxin B subunit (LTB) with an in-house multiplex immune assay, because this toxin is highly immunogenic and contributes to the diarrhea following ETEC infection. However, out of 19 volunteers, only 6 had a greater than twofold IgG and 4 had a greater than twofold IgA response against LTB (unpublished results). The rather low anti-LTB response accorded with what has been observed in previous studies,³⁵ and because of the restricted amounts of salivary fluids available, we decided not to include this antigen in our tests.

The increase in total IgA and SIgA concentration in submandibular/sublingual and parotid secretions as well as in the intestinal lavage fluid observed after challenge is notable. The absolute amount of specific salivary IgA was low and could not alone explain the observed elevation of total salivary IgA. In intestinal lavage, the increase could have reflected a response to the experimental intestinal ETEC infection combined with irritation by the laxative treatment. However, even when normalizing the specific SIgA anti-ETEC against the total SIgA concentration, the specific responses remained convincing.

One possibility for the total IgA response after ETEC challenge is that the immune response elicited in GALT by the experimental ETEC infection might have triggered bystander activation of B cells, for instance induced by lipopolysaccharide, which is a known B cell mitogen; this could have not only stimulated the dissemination of specific B cells but also induced polyclonal responses giving rise to antibodies of unknown specificities (“natural antibodies”) in a T-cell-dependent or -independent manner.^{36,37} Studies in mice have shown that intestinal IgA elicited by exposure to one particular new commensal bacterium in the gut may contain less than 10% detectable specific antibodies.³⁸ Experiments in one of our laboratories with focused mucosal chronic stimulation by a single protein antigen (egg white lysozyme) resulted in the induction of at least 60% plasma cells of unknown reactivity colocalized with the specific cells at the same site.³⁹

Normalization of specific IgA against total IgA in specimens collected from various mucosal sites is often used to correct for variation of flow rate and/or eventual dilution effects during sampling. The rationale for this approach is obvious in specimens such as fecal extracts and nasal washes, whereas it is more questionable regarding sampling of (non-stimulated) salivary secretions as discussed in our review of methodological problems jeopardizing the quantification of IgA in saliva.²⁰ When total IgA increases as a consequence of mucosal immune stimulation, normalizing against total IgA will reduce, and sometimes even mask, the specific response. Although the secretion rate of parotid IgA in an individual appears to be more stable over time than the actual IgA concentration,^{40,41} SIgA is more subjected to short-term variation than other salivary proteins.^{42,43} This may not only reflect differences in the glandular structures involved in SIgA generation, but it is also reflecting that salivary IgA is a product of adaptive immunity. The average density of IgA-producing cells is considerably

higher in human submandibular than parotid glands,⁸ and studies in inbred mice have suggested that fluctuations in glandular IgA⁺ plasmablasts/plasma cells contributes to variations in salivary IgA levels.⁴⁴

Standardized IgA quantitation in whole saliva is quite challenging.²⁰ It has been estimated that the minor salivary glands contribute 30–35% of the total salivary IgA, while sublingual/submandibular and parotid glands contribute about equal amounts.⁴⁵ Whole-saliva samples usually require centrifugation before quantification, and the sediment represents a variable IgA loss, probably because SIgA binds not only to mucus^{46,47} but also to oral bacteria.⁴⁸ More controlled sampling is obtained by using absorbing sponge (pads) such as Salimetrics (State College, PA) used in the present study.

It is not well delineated which part of mucosa-associated lymphoid tissue is most important for induction of immune responses subsequently expressed as salivary IgA antibody, but there is convincing evidence both in animals and in humans that activated B cells can migrate from GALT to salivary glands.^{49,50} Nevertheless, in subjects immunized orally with a cholera toxin B subunit–whole-cell *Vibrio cholerae* vaccine, the specific IgA antibody detection sensitivity in saliva (without normalization of the specific levels) was not better than in serum and only about 50% of that in intestinal lavage. After infection with *V. cholerae* or enterotoxigenic *E. coli*, the detection sensitivity for antibodies against the respective toxins increased, but not as much as that was seen in serum, suggesting considerable leakage of plasma IgA into the oral cavity.¹⁶ Also notably, that study probably overestimated the enteric–oral B-cell homing axis because cholera toxin and enteropathogens breach the gut mucosa to become unduly disseminated to the systemic immune system, as reflected by the high levels of specific serum IgG antibodies. Thus, intestinal immune induction was not well reflected in the salivary IgA system, a conclusion reached also in several other studies.^{13–15,18}

We found a substantial increase in specific IgA in saliva against the challenge ETEC strain from pre-challenge to post enteric challenge, most significant at day 10. Both the salivary and the serum IgA response peaked at day 10 with a subsequent decline at day 28, whereas strain-specific serum IgG antibodies appeared to be relatively high even at day 28. Although the GM rise in IgA titer against the challenge strain was only marginally greater in the sublingual than in the parotid fluid, the proportion of volunteers who responded with more than a twofold increase in the levels of anti-bacterial IgA was significantly larger when assessed with the sublingual samples. Considering that there must be some admixture of parotid secretions into the fluid collected underneath the tongue, these differences are likely to represent an underestimation of the disparate response patterns. Our findings therefore support the original hypothesis for the design of this study—namely that IgA plasma cell precursors generated in GALT home more consistently to sublingual/submandibular than to parotid glands. Although we have not directly shown this possibility, it is the most likely the explanation for our observation that

secretion from the former glands reflects intestinal immune induction better than parotid secretion.

Altogether, our study demonstrates that IgA antibodies in targeted secretions collected from underneath the tongue, rather than in parotid secretions or whole saliva admixed with the latter, may serve as a useful noninvasive readout with high detection sensitivity for intestinal immune induction after experimental ETEC infection in healthy young adults. It remains to be shown whether this result can be reproduced in different age groups and after enteric vaccination.

METHODS

Ethics approval. The study was approved by the Regional Committee for medical and Health Research Ethics, Health Region West (REC-West, case number 2010/728-14). The subjects provided their informed written consent to participate in the study.

Infection study and sample collection. The infection study is detailed in a previous paper.²⁷ Briefly, 30 healthy adult volunteers were infected *per os* with wild-type enterotoxigenic *E. coli* (ETEC) strain TW10598 in doses ranging from 1×10^6 to 1×10^9 colony-forming units. Targeted and simultaneous sampling of unstimulated sublingual/submandibular parotid secretions was performed with oral swabs (Salimetrics) placed under the tongue and between the alveolar arch and the buccal mucosa at the opening of Stenson's ducts on both sides for 5 to 10 min, respectively. The swabs were centrifuged at 1,800 g for 10 min to expel the secretion. Some samples provided relatively small amounts of fluid, and sterile saline was therefore added up to make up a volume of 1.9 ml, the dilution factor being noted. Salivary secretions and blood samples were collected before infection (day 0), and at day 7, 10 (median 10, range 9–10), and 28 (median 27 days, range 16–36 days) after experimental infection. Intestinal lavage was induced by the intake of Laxabon (Recipharm Höganäs AB, Höganäs, Sweden) according to the manufacturer's guidelines. To minimize the risk of IgA degradation in the lavage fluid, protease inhibitor (Complete, mini, EDTA-free protease inhibitor, Roche, Diagnostics Norge, Oslo, Norway) was added according to the manufacturer's instruction and the specimens were aliquoted and frozen immediately at -20°C .

ETEC strains and growth conditions. Specific antibody responses were measured against live ETEC bacteria of the challenge strain and against a heterologous ETEC strain. Both strain TW10598 (serotype O6:H16)⁵¹ and strain H10407 (serotype O78:H11) were grown on Colombia agar plates overnight. Colonies were collected and suspended in Hank's balanced salt solution (HBSS) supplemented with 2 mg ml^{-1} bovine serum albumin.

Antibodies in lymphocyte supernatant (ALS). The ALS was prepared as previously described,^{52,53} with the exception that peripheral blood mononuclear cells were isolated from cell preparation tubes (Becton, Dickinson and Company, Franklin Lakes, NJ) and washed initially in phosphate-buffered saline followed by Hank's buffered saline (HBSS). Peripheral blood mononuclear cells were counted with the aid of a hemacytometer and adjusted to a concentration of 1×10^7 cells in complete RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine and $50\text{ }\mu\text{g ml}^{-1}$ gentamicin sulfate peripheral blood mononuclear cells in medium (1.5 ml per well) were plated into 24-well Nunclon Delta Surface plates (Thermo Scientific, Oslo, Norway) and incubated for 72 h at 37°C with 5% CO_2 . Following incubation, lymphocyte supernatants were collected, aliquoted, and stored at -80°C until they were assayed by flow cytometry for antibodies against the ETEC challenge strain.

Determination of specific antibodies against the ETEC strains. Specific antibodies against the ETEC strains were measured by flow cytometry with live bacteria,⁵⁴ which were grown overnight, harvested,

washed in HBSS/bovine serum albumin, and adjusted to an optical density of 0.7 at 650 nm. The specimens were analyzed at twofold dilutions in HBSS/bovine serum albumin in U-bottomed 96-well microplates (Nunc A/S, Roskilde, Denmark). For antibody determination of IgA in salivary secretions, intestinal lavage, and ALS, as well as of IgA and IgG antibodies in serum, a serum sample from one of the participants with high IgA and IgG antibody levels against the challenge strain TW10598 was included in each plate to construct the standard curve. For determination of SIgA in saliva and intestinal lavage fluid, a lavage sample with high levels of SIgA antibodies was used to prepare the standard curve in AUs: 50 μ l dilution of test specimen and 5 μ l bacteria were mixed and incubated for 45 min at 37 °C, and the bacteria were washed twice by centrifugation and incubated with 1/100 dilution of phycoerythrin-conjugated anti-IgA (2050-09, Southern Biotech, Birmingham, AL) or a phycoerythrin anti-IgG (P8047, Sigma-Aldrich, Oslo, Norway) for 45 min. For SIgA determination, a monoclonal antibody (I6635, Sigma-Aldrich) against SC was used at 1/500 dilution, and thereafter detected with a goat-anti-mouse IgG fluorescein isothiocyanate-labeled (F0479, DAKO, Glostrup, Denmark) at 1/40 dilution. For detection, IgA subclass antibodies in salivary secretions and in intestinal lavage, murine monoclonal antibodies to IgA1 (clone 69-11.4 ascites) and IgA2 (clone 16-512-H5 ascites) at 1/2000 dilution were used,³² followed by the fluorescein isothiocyanate-labeled goat-anti-mouse IgG. Finally, the bacteria were washed and suspended in 100 μ l HBSS/bovine serum albumin supplemented with 10 μ g ml⁻¹ of Hoechst 34580 (Life Technologies, Oslo, Norway). The fluorescence intensity reflecting bound antibodies was read by an Attune flow cytometer (Life Technologies), gating on the bacteria with combined side scatter/Hoechst 34580 fluorescence. Some experiments were run on a CyFlow ML (Partec, GmbH, Munster, Germany) flow cytometer; in that case, the bacteria were counter-stained with SYTO 61 (Life Technologies). The standard curve was drawn from the GM phycoerythrin or fluorescein isothiocyanate fluorescence intensity of the various dilutions of the standard serum or standard lavage fluid in AU per ml. The GM phycoerythrin or fluorescein isothiocyanate fluorescence intensity from the samples were interpolated on the standard curve with GraphPad Prism (La Jolla, CA) software version 5.

All experiments were repeated two or three times, and all samples collected at different time points from one subject were analyzed on the same plate. Data from one representative experiment are presented.

Determination of total IgA concentration in saliva and intestinal lavage. Total IgA in salivary secretions and lavage was determined by an enzyme-linked immunosorbent assay. Microplates (Nunc MaxiSorp, Roskilde, Denmark) were coated with a rabbit anti-human IgA antibody (I9889, Sigma-Aldrich) at 1/2,000 dilution in phosphate-buffered saline with 0.02% azide and stored for 2–30 days at 4 °C until use. Before adding the samples, the coating solution was removed and the plates were incubated with blocking buffer of 0.5% (w/v) gelatin in phosphate-buffered saline for 30 min. Salivary and intestinal lavage specimens were diluted twofold, starting with 1/2,000 in phosphate-buffered saline containing 0.1% gelatin and 0.05% Tween 20. Twofold dilutions of human colostrum (I2636, Sigma-Aldrich) with a defined concentration of IgA were used to make the standard curve. After a 90-min incubation and a washing step, an anti-human IgA alkaline phosphatase conjugate (A9669, Sigma-Aldrich) diluted 1/20,000 was added and incubated for 90 min. Finally, the substrate p-nitrophenyl phosphate was added and incubated for about 40 min. All incubations were performed at 37 °C. The absorption was read at 405 nm with a BioTek EL808 (Winooski, VT) microplate reader, and the Gen5 software was used to draw the standard curve (four-parameter non-linear regression) and to interpolate the unknown samples. As an internal marker of fluid flow rate, albumin was measured in the salivary samples with a commercial ELISA kit (ab108788 Albumin Human ELISA kit, Abcam, Cambridge, UK) according to the manufacturer's instruction.

Statistical analyses. GM concentration and 95% CI of total IgA and specific antibodies were calculated at each time point. Linear regression analysis of log-transformed data was used to describe relationships between different readouts. Differences in log-transformed titers and between the proportion of volunteers with more than twofold increase in such titers between various time points were examined by two-sided *t*-tests and McNemar's exact chi-square test, respectively.

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DISCLOSURE

The authors declare no conflict of interest.

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