Measurement of Faecal Immunoglobulin A Levels in Young Children

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A nephelometric immunoassay was developed to quantify immunoglobulin A (IgA) in children's stools. This method enables IgA in faecal protein extracts to be measured over a large range of concentrations (1.61–51.50 mg/L) with good accuracy (linear recovery in dilution-overloading assay) and precision (within- and between-run coefficients of variation (CVs) of 1–6%). An excellent recovery (105%) was obtained in stool samples overloaded by purified colostral IgA, demonstrating that the method used for faecal IgA extraction is adapted, not to induce significant IgA degradation, and probably allow a complete extraction of

IgA. The amount of faecal IgA, as determined in stool samples from 125 children (6–24 months old), was an average of 14 mg per 100 g of stools (about 10% of the total protein stool content), with large individual variation (3–30 mg per 100 g of stools). No correlation was observed between faecal IgA amounts and the children's age or sex. Such an immunoassay could enable exhaustive noninvasive investigations of the maturation of the intestinal immune system, as well as accurate studies of the effect of oral dietary supplementation on IgA regulation. *J. Clin. Lab. Anal.* 18:195–199, 2004. © 2004 Wiley-Liss, Inc.

Key words: immunoglobulin A; stools; immunoassay

INTRODUCTION

The first observations suggesting that intestinal mucosae possess their own humoral immune system were published in the beginning of the 20th century (1,2), in the pathological context of typhoid fever or dysentery. By the mid 1960s, this system had been well characterized in adult subjects (3), and immunoglobulin A (IgA) was identified as the predominant immunoglobulin isotype of this compartment. It was then shown that the IgA produced by mucosal plasma cells are polymeric (4), especially in the gut, and that the poly-Ig receptor that provides them with their definitive molecular structure is acquired within epithelial cells during the phenomenon of transcytosis (5). Secretory IgA (SIgA) can bind to antigens, such as microorganisms and microparticles, and prevent their adhesion to or penetration into the lamina propria (6,7). The estimated daily intestinal production of SIgA in human adults is about 40 mg/kg body weight/day (8). These IgA are entirely produced by the mucosae-associated lymphoid tissue (MALT (9), and transcytose to the lumen. According to Meillet et al. (10), the SIgA normally found in faeces result from the cumulated secretion of saliva, bile, and intestinal fluid, as human

SIgAs are fairly resistant to both acid and proteolytic enzymes (4,11,12). SIgA is absent in children at birth, and becomes detectable in mucosal secretions at the ages of 1 week to 2 months (13–15). The appearance of specific SIgA in children is dependent on natural exposure to environmental antigens (16). The bacterial, viral, or parasitic infections of childhood are associated with an increased rate of IgA production in response to antigenic stimulation. Consequently, mucosal IgA levels and specificity constitute a good marker of immunological events in the digestive tract (17). However, very few studies have examined the maturation of digestive IgA production in children (15).

In this work we report the development of a nephelometric immunoassay for faecal IgA, and its application to 125 stool samples from healthy young children.

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MATERIALS AND METHODS

Faecal Extracts

Fresh stool samples were collected from diapers, rapidly snap-frozen in liquid nitrogen, and stored at -80°C until tested. The protocol used to extract protein from the stool samples was derived from those described by Meillet et al. (10) and Warny et al. (18). Each frozen faecal sample was weighed and then rapidly thawed while it was resuspended and homogenized under gentle agitation in 1 M phosphate buffer containing 0.14 M NaCl, 2 mM phenyl-methyl-sulfonyl-fluoride as protease inhibitor, and 1 mM NaN_3 as bacteriostatic agent. The final volume was adjusted at 25 mL and each specimen was immediately submitted to a first centrifugation at 1,200 g for 15 min at 4°C , followed by a further centrifugation at 8,000 g for 30 min at 4°C . The clear supernatant was collected, distributed in 1.5-mL aliquots, and stored at -80°C .

The total protein concentration of each faecal extract was determined by a micro Lowry method (19), and referred to the initial weight of each stool sample.

Faecal IgA Immunoassay

We performed a nephelometric immunoassay of faecal IgA as a one-step test in a reaction microcuvette (Nephelia[®]; Sanofi-Diagnostics-Pasteur, Marnes la Coquette, France) by mixing 100 μL of faecal extract or diluted (1/40 to 1/1280) calibrator (Behring Diagnostics, Marburg, Germany) with 40 μL of anti-human IgA α -chain rabbit antiserum (Behring) and 160 μL of 0.14M NaCl. The scattered light was measured with a Sanofi-Diagnostics-Pasteur Nephelometer Nephelia[®] N600 after the samples were incubated for 30 min at room temperature. The results, expressed in mg/L of faecal extract, were then referenced to the initial weight of stool (mg/100 g of stool).

Precision and Analytical Recovery of the Faecal IgA Immunoassay

We assessed the precision of the faecal IgA immunoassay by measuring IgA in faecal extract samples with low, intermediate, and high concentrations, 10 times during the same assay (for within-run precision) and in 10 successive assays (for between-run precision).

The IgA recovery in faecal extract was tested in a dilution-overloading experiment. The dilution assay was performed on serial dilutions of two faecal extract samples containing 23.97 mg/L (1/2–1/8) and 14.50 mg/L (1/2 and 1/4), respectively, of IgA. For the overloading assay, four different increasing amounts (10.8–43.2 mg/L) of purified colostral SIgA (Sigma Chemical Co., St. Louis, MO) were added to one faecal extract sample

(3.32 mg/L). The slopes calculated by linear regression analysis for the dilution and the overloading assays were compared with the use of a Student's *t*-test. For the total recovery, including dilution and overloading assays, the null hypothesis H_0 (intercept = 0; slope = 1) vs. the alternative hypothesis H_1 (intercept $\neq 0$; slope $\neq 1$) were tested by means of *F*- (Fisher) and *t*-tests, respectively.

Recovery of IgA in Overloaded Stools

Ten stool samples were thawed and homogenized in 8 mL of 1 M phosphate buffer containing 0.14 M NaCl, 2 mM phenyl-methyl-sulfonyl-fluoride, and 1 mM NaN_3 . Each suspension was separated in two aliquots of 4 mL. One of these aliquots was overloaded with 60 μg of purified colostral SIgA (Sigma). All of the samples, overloaded or not, were then treated as described above to obtain faecal protein extracts. The concentration of IgA in the 10 extracts of native stools and the 10 extracts of overloaded stools was measured by immunonephelometric assay, and the recovery of added IgA was calculated for each overloaded stool.

Application to Infant Stool Samples

The immunoassay of faecal IgA was applied to stool samples collected from 125 healthy young children (61 girls and 64 boys, age range = 6–24 months, mean = 14.5 months). The procedures followed were in accordance with the ethical standards of the responsible national committee on human experimentation.

The concentrations of IgA measured in each faecal extract were referred to the initial weight of the stool sample (~ 3 g). The ratio of IgA concentration to total protein concentration was computed for each stool. The computer program GraphPad Prism 3.00 (GraphPad Software, San Diego, CA) was used for all of the data and statistical analyses. The Gaussian distribution of the population was tested by a Kolmogorov-Smirnov test. The Pearson correlation between the IgA content of each stool and the child's age was investigated. Possible relationships between the children's sex and the IgA content of the stool were tested by means of a Student's *t*-test.

RESULTS

Immunoassay of Faecal IgA

A calibration range of 1.61–51.50 mg/L of IgA in faecal extracts (Fig. 1) was obtained when the assay was performed as described in Materials and Methods.

The precision of the faecal IgA immunoassay was assessed by the coefficients of variation (CVs) obtained in within- and between-run studies (0.6–5.3%) over a large range of concentrations, as shown in Table 1.

Fig. 1. Calibration curve for the faecal IgA immunoassay. The assay procedure is described in the text.

Fig. 2. Analytical recovery of faecal IgA. ■, ♦ and , dilution assay; △ and ----, overloading assay; ———, linear regression for total recovery. Experimental details and linear parameters are given in the text.

TABLE 1. Precision of faecal IgA determination

	Within-run precision (n = 10) ^a			Between-run precision (n = 10) ^a		
Min (mg/L)	7.63	11.55	43.16	7.11	10.51	41.61
Max (mg/L)	8.17	12.49	45.02	7.46	12.43	42.34
Mean (mg/L)	7.78	11.98	43.80	7.30	11.30	41.94
SD ^b (mg/L)	0.17	0.31	0.63	0.12	0.60	0.26
CV ^c (%)	2.2	2.6	1.4	1.7	5.3	0.6

^an = number of assays.

^bStandard deviation.

^cCoefficient of variation.

The analytical recovery of the faecal IgA immunoassay (Fig. 2) was linear ($n = 12$, $r = 0.999$, $P < 0.001$) for the range of IgA concentrations in faecal extract tested in dilution-overloading assays (3.00–46.52 mg/L). A mean percentage of recovery of 100.4% was obtained for IgA in the overloaded faecal extracts. The slopes of the dilution (0.944) and overloading (0.997) curves were not significantly different ($P > 0.05$). The slope (0.996) and the intercept (0.149 mg/L) of the total recovery curve, including the dilution and overloading assays, were not significantly different ($P > 0.05$) from 1 and 0, respectively.

When purified colostral IgA was added to 10 children's stools before protein extraction, a mean percentage of recovery of 104.6% (SD = 6.7%) was observed for IgA in overloaded stools.

Application to Infant Stool Samples

The application of the IgA immunoassay to stool samples from 125 children yielded the following global results: minimum = 3.29 mg/100 g of stools, maximum = 30.44 mg/100 g of stools, mean = 13.90 mg/100 g of stools, and SD = 6.76 mg/100 g of stools. The ratio of IgA on total protein in faecal extracts ranged from 21.7 to 322.9 $\mu\text{g}/\text{mg}$ (mean = 97.6 $\mu\text{g}/\text{mg}$; SD = 30.24 $\mu\text{g}/\text{mg}$).

No correlations were observed between the amount of IgA in the stool samples and the age or sex of the children.

DISCUSSION

The nephelometric immunoassay reported here was developed to determine the amount of IgA in children's stools. It can be performed easily (in a one-step assay, without washing or phase separation) and rapidly (30 min), and thus allows the measurement of IgA concentrations in large series of samples. The concentration of IgA in faecal extracts is measured over a large range (1.61–51.50 mg/L) with high reproducibility (CVs < 6% in within- and between-run precision) and accuracy (linear recovery in a dilution-overloading assay).

The good recovery (105%) of stool overloaded by purified colostral IgA demonstrates that the method

used for faecal IgA extraction is adapted not to induce significant IgA degradation, and probably allow for a complete extraction of IgA. An SDS-PAGE gel analysis under nondenaturing conditions confirmed the integrity of SIgA in faecal extracts and overloaded stools (data not shown).

IgA assays have been reported to be difficult to perform in intestinal secretions, and have mostly been performed for patients with inflammatory bowel disease, Crohn's disease, or coeliac disease, often by invasive collection methods. Meillet et al. (10) measured IgA production after in vitro culture of biopsy material. Another method, which is supposed to provide the best accuracy, consists of measuring IgA in whole-gut lavage fluid (20) obtained after oral administration of large volumes of polyethylene glycol (PEG)—a procedure that is not applicable to young children. However, several previous studies showed that it is also possible to quantify IgA directly in stool samples (10,21,22), and the authors concluded that the amount of IgA in small faecal samples appears to be a good reflection of the total quantity of IgA secreted by the gut. This method is completely noninvasive; however, the presence of digestive enzymes and proteases produced by microorganisms of the flora may be responsible for the denaturation of IgA in stools (10). This imposes strict collection, storage, and treatment protocols, as were applied here. In the current study, SDS-PAGE under nondenaturing conditions displayed high molecular mass IgA corresponding to SIgA, thus assessing the dimetric integrity of these macromolecules in our faecal extracts. Similarly, the absence of detectable serum albumin jointly measured in children's stools demonstrated the local origin of the IgA assayed (data not shown).

This study shows that in healthy young children, the faecal amount of IgA is an average of 14 mg per 100 g of stools (about 10% of the total protein stool content), with large individual variation (3–30 mg per 100 g of stools). These values are comparable to faecal IgA amounts (7–66 mg per 100 g of stools) previously determined by other authors (10,22), and to IgA levels measured in gut lavage fluid (14–33 mg/100 mL (23) or jejunal fluid (3–18 mg/100 mL) (24). This suggests that a near-normal production of IgA is achieved in at least a good proportion of children before 2 years of age. The large individual variations observed in this study could be related to the faecal water content and/or the intestinal transit time, which might favour in vivo destruction of SIgA by digestive enzymes and bacterial proteases (20). They could also be related to individual differences in MALT maturation.

The immunoassay of faecal IgA developed in this work may enable exhaustive investigations of the

maturation of the intestinal immune system over age, as well as the possible effects of oral dietary supplementation on this maturation process.

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