ORIGINAL

Measurement of allergen-specific secretory IgA in stool of neonates, infants and toddlers by protection against degradation of immunoglobulins and allergens

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Abstract : Background and aims : Measurement of secretory immunoglobulin A (SIgA) level is important to monitor various disease conditions. However SIgA in gut mucosa is degraded by pancreatic proteases and proteolytic enzymes from enteric microbiota. Currently, there is no reliable quantitation method that measures allergen-specific SIgA levels in stool of neonates, infants and toddlers. Methods : Allergen-specific SIgA levels in stool of 10 healthy neonates, infants and toddlers aged 0 to 36 months were measured by our new allergen microarray with densely carboxylated arms on a glass slide chip. Results : Protease activities in stool of 3-day-old neonates were low and no degradation of SIgA, IgA and allergens was detected. However, immunofluorescence signals of SIgA, IgA and allergen on the chip were markedly reduced by stool extracts obtained from infants and toddlers aged more than one month in dose- and time-dependent manners. Such reduction was almost completely inhibited by serine protease inhibitors, phenylmethylsulfonyl fluoride (PMSF) and partly by leupeptin, but not by a variety of other protease inhibitors tested. Conclusion : Allergen-specific SIgA levels in stool of neonates, infants and toddlers under 36 months of age could be analyzed using protease inhibitors, including PMSF and leupeptin. J. Med. Invest. 62 : 137-144, August, 2015

Keywords **:** *allergen microarray ; stool SIgA ; SIgA degrading enzyme ; serine protease inhibitor*

INTRODUCTION

The incidence of food allergies has increased significantly in the past decades and is currently a worldwide public health problem. Gastrointestinal food allergies in neonates, infants and young children are due to developmental immaturity of various components of the gut barrier and gut immune system (1) and exhibit a variety symptoms and disorders involving the skin and gastrointestinal and respiratory tracts (2).

The gut-associated lymphoid tissue organized in solitary and aggregate follicles within the gut plays a central role in defense against many invading pathogens and the development of immune-tolerance against food allergy (3-7). The immune system is mainly mediated through B cells, producing secretory IgA (SIgA) which acts as a blocking or neutralizing antibody (8-12). To monitor the immunological maturity of the gut, it is important to measure antigen- or allergen-specific SIgA levels in stool of patients with gastrointestinal food allergies and assess immunotolerance of allergy in the mucosa (13, 14). For accurate quantitative measurement of SIgA in stool, the roles of pancreatic enzymes should be taken into consideration, such as trypsin, chymotrypsin (15, 16), and various immunoglobulin-degrading proteases, such as IgA_1, IgA_2, SIgA or IgG proteases, in *Escherichia coli* (17, 18) and other microbiota in stool.

The present study is an extension to our previous study on our new sensitive allergen-microarray for monitoring various immunoglobulin parameters in serum and saliva (19-23), and was designed to detect SIgA in stool. Specifically, we used the densely carboxylated protein chip (DCP chip), with modified carboxylated arms on the surface of the diamond-like carbon-coated chip (19) or the surface of the glass slide chip. In this study, we used the glass slide DCP chip to detect allergen-specific SIgA in stool.

MATERIALS AND METHODS

Materials

Phenylmethylsulfonyl fluoride (PMSF), leupeptin, Bestatin, E-64c, aprotinin, chymostatin and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma (St. Louis, MO). Protease substrates, *t*-butyloxycarbonyl (Boc)-L-Phe-Ser-Arg 4-methylcoumaryl-7-amide (MCA), Leu-MCA, benzyloxycarbonyl (Z)-L-Phe-Arg-MCA and (7-methoxycoumarin-4-yl) acetyl (MOCAc)-L-Arg-Pro-Lys-Pro-Tyr-Ala-norvalyl (Nva)-Trp-Arg-N^{ε}-(2,4-dinitrophenyl)-L-Lys (Lys(Dnp))-NH2 were purchased from Peptide Institute (Osaka, Japan).

Sample collection

Stool samples were collected from 10 healthy neonates, infants and toddlers (age, 0-36 months), and immediately treated with or without protease inhibitors, then stored at -30°C until use. Stool samples were also collected separately from another group of 20 one-month-old infants for analysis of the relationship between serine protease activities and protease-mediated degradation of IgA and SIgA. The mother of each neonate/infant/toddler gave informed consent to sample collection. The study was approved by the ethics committees of the National Research Institute for Child

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Health and Development, and Tokushima University Hospital.

Measurement of allergen-specific SIgA

We used a glass slide DCP chip (Gene slide ; Toyo Kohan Co. Tokyo, Japan) instead of a diamond-like carbon-coated DCP chip (19) in this study to measure allergen-specific SIgA levels and their immunoresponse profile.

Purified molecular allergens, including ovomucoid, ovalbumin, conalbumin, α-casein, β-casein, κ-casein, α-lactoalbumin and β-lactoglobulin, were purchased from Sigma-Aldrich (St Louis, MO). The allergen solutions of ovomucoid (1 mg/ml), ovalbumin (0.5 mg/ml), conalbumin (1 mg/ml), α-casein (1 mg/ml), β-casein (1 mg/ml), κ-casein (0.25 mg/ml), α-lactoalbumin (1 mg/ml) and β-lactoglobulin (1 mg/ml) in 4.4 nl, were spotted tetraplicates and immobilized by covalent binding on the chip. Human IgA (67/086), as an internal standard on the chip, was obtained from the National Institute for Biological Standards (Hertfordshire, UK). The allergen microarrays were incubated with 20 μl of stool extracts for 60 min at 37°C, and then SIgA antibodies bound on each allergen was visualized by HiLyte Fluor 555 (Dojindo Molecular Technologies, Kumamoto, Japan)-labeled secondary antibody (Life Technologies, Gaithersburg, MD) against human IgA for 60 min at 37°C, which reacts both SIgA and IgA. The immunofluorescence intensity of each allergen was analyzed using FLA-8000 scanner (Fujifilm Co., Tokyo) with an excitation wavelength of 532 nm and emission wavelength of 570 nm under 60% impressed voltage, as described previously (19-23).

Stool sample extraction

Stool samples (100 mg) in extraction buffer (5 ml) of the EDN ELISA Kit (MBL, Nagoya, Japan) (24), were suspended for few min and then insoluble materials were removed by centrifugation at 13000×*g* for 5 min at 4°C. The supernatant was stored at -30°C until use. Stool extracts were diluted to 0.25 mg protein/ml using phosphate-buffered saline (PBS), containing 1% bovine serum albumin (BSA), 0.3 M KCl and 0.05% Tween 20. The levels of allergen-specific SIgA in the stool extract were analyzed on the glass slide DCP chip.

Measurement of activities of various proteolytic enzymes in stool extracts

The activities of various proteolytic enzymes in the stool extracts were measured using the modified methods described previously (25-28). The activity of trypsin-type protease was measured by the hydrolysis of 20 μM Boc-Phe-Ser-Arg-MCA in 0.1 M Tris-HCl buffer, pH 8.0, at 37°C for 10 min. The activity of amino-peptidase was monitored by the hydrolysis of 50 μM Lue-MCA in 0.1 M Tris-HCl buffer, pH 7.4, at 37°C for 10 min. The activity of cysteine protease was assayed by the hydrolysis of 20 μM Z-Phe-Arg-MCA in 0.1 M acetic buffer, pH 4.0, containing 10 mM cysteine at 37°C for 10 min. The activity of metalloprotease was analyzed by the hydrolysis of 10 μM MOCAc-Arg-Pro-Lys-Pro-Tyr-Ala-Nva-Trp-Met-Lys (Dnp)-NH_2 in 0.1 M Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl, 10 mM $CaCl_2$ at 37°C for 10 min. The enzyme activity was measured as fluorescence (excitation at 370 nm, emission at 460 nm) using a plate reader (Total reaction volume is 200 μl, Spectra max GEMINI EM 200, Molecular Devices, Sunnyvale, CA). One unit of enzyme activity was defined as the amount of the activity that released 1 μmol of MCA from the substrate per min.

Reduction of allergen immunoreactivity by stool samples

Stool extracts were applied onto the allergen chip, incubated at 37°C for 60 min and then washed. Pooled sera containing allergen-specific IgA from allergic patients were applied on the chip to measure the immunoreactivity of allergens, incubated at 37°C for 60 min and then washed. The levels of allergen-specific SIgA were then analyzed by IgA immunofluorescence intensity.

Reduction of immunoreactivity of allergen-specific SIgA by stool protease(s)

To analyze the stability of allergen-specific SIgA in stool, stool extracts were prepared as follows. Stool extracts were sonicated with 30 bursts of 15 sec at 4°C using bath-type sonicator (Bioruptor UCW-310, Cosmo Bio Co., Tokyo), and incubated by flopping upside down at 4°C for 60 min. Next, the insoluble materials were removed by centrifugation at 17400×*g* at 4°C for 10 min. After filtration of the supernatant through 0.45 μm pore-size filter (Kurabo, Osaka, Japan), the filtrates were incubated for the indicated time intervals (from 0 to 24 hr). After incubation, the activities of various proteases in stool were stopped by the addition of protease inhibitor cocktail containing 10 mM PMSF, 0.1 mM leupeptin, 0.1 mM bestatin and 0.1 mM E-64c in PBS buffer containing 15% dimethylsulfoxide, 0.3 M KCl and 0.05% Tween 20. The concentration of proteins in the extracts was then adjusted to 2.5 mg/ml by PBS containing 10% BSA, 0.3 M KCl and 0.05% Tween 20. After the reaction, the stool extracts were incubated with allergen-microarray for 60 min and the immunofluorescence intensity of allergen-specific SIgA bound on each allergen was measured by a HiLyte Fluor 555-labeled secondary antibody after further incubation for 60 min at 37°C.

Statistical analysis

Statistical analysis was performed using SPSS software (SPSS, Chicago, IL, USA). Paired correlations were analyzed for significance by the spearman rank test.

RESULTS

Activities of various protease in stools of neonates, infants and toddlers

The gastrointestinal tract of neonates, infants and toddlers undergoes marked structural and functional adaptation in response to feeding and the rapid and large changes are characterized by induction of digestive proteases in the gastrointestinal tract, development of intestinal microbiota and mucosal immunity. All such changes should be reflected in stool samples. We analyzed the proteolytic activities of serine proteases, cysteine proteases, aminopeptidases and metalloproteases in stool samples of neonates, infants and toddlers aged 0-28 days, 6-7 months and 1-3 years (Table 1). The activities of serine proteases and metalloproteases in stool extracts increased markedly to about 8 to 9 folds and 6 to 7 folds, respectively, at the weaning period of 6-7 months of age and the levels were still high at 1-3 years of age. On the other hand, the activities of cysteine proteases and amino-peptidases were only slightly increased (to about 2-fold) at the weaning period (Table 1).

Stool samples from 1-month-old infants but not 3-day-old neonates reduce IgA immunofluorescence signals

Although relatively low levels of protease activities were detected in stool samples of neonates and infants, we examined the effects of stool proteases on IgA, allergen-specific SIgA and allergens on the chip.

To analyze the levels of milk and egg allergen-specific SIgA in the stool, molecular allergens and various doses of WHO human IgA standards (0.05-10 IU/ml) were spotted in tetraplicates on the glass DCP chip and immobilized as described previously (19) (Fig. 1A). After incubation with stool extracts on the chip for various time periods (0-60 min), SIgA captured on allergens and standard IgAs were visualized with HiLyte Fluor™ 555 labeled anti-human IgA with rainbow display. To our surprise, no allergen-specific SIgA signal was detected with stool extract of 1-month-old infants and

Table 1. Activity of various proteases in stools of neonates, infants and toddlers.

	Enzyme activity			
	Serine protease (mU/μg)	Cysteine protease (mU/μg)	Amino peptidase (mU/μg)	Metalloprotease (FLR/μg)
0-28 days (n=3)	17.0±4.6	0.08±0.04	3.9±3.1	466±485
6-7 months (n=3)	154±73	0.16±0.03	9.8±5.9	3411±1141
1-3 years (n=4)	143.2±87.4	0.11±0.05	14.0±6.1	2593±662

Values are means±SD of three or four separate stool samples of neonates, infants and toddlers.

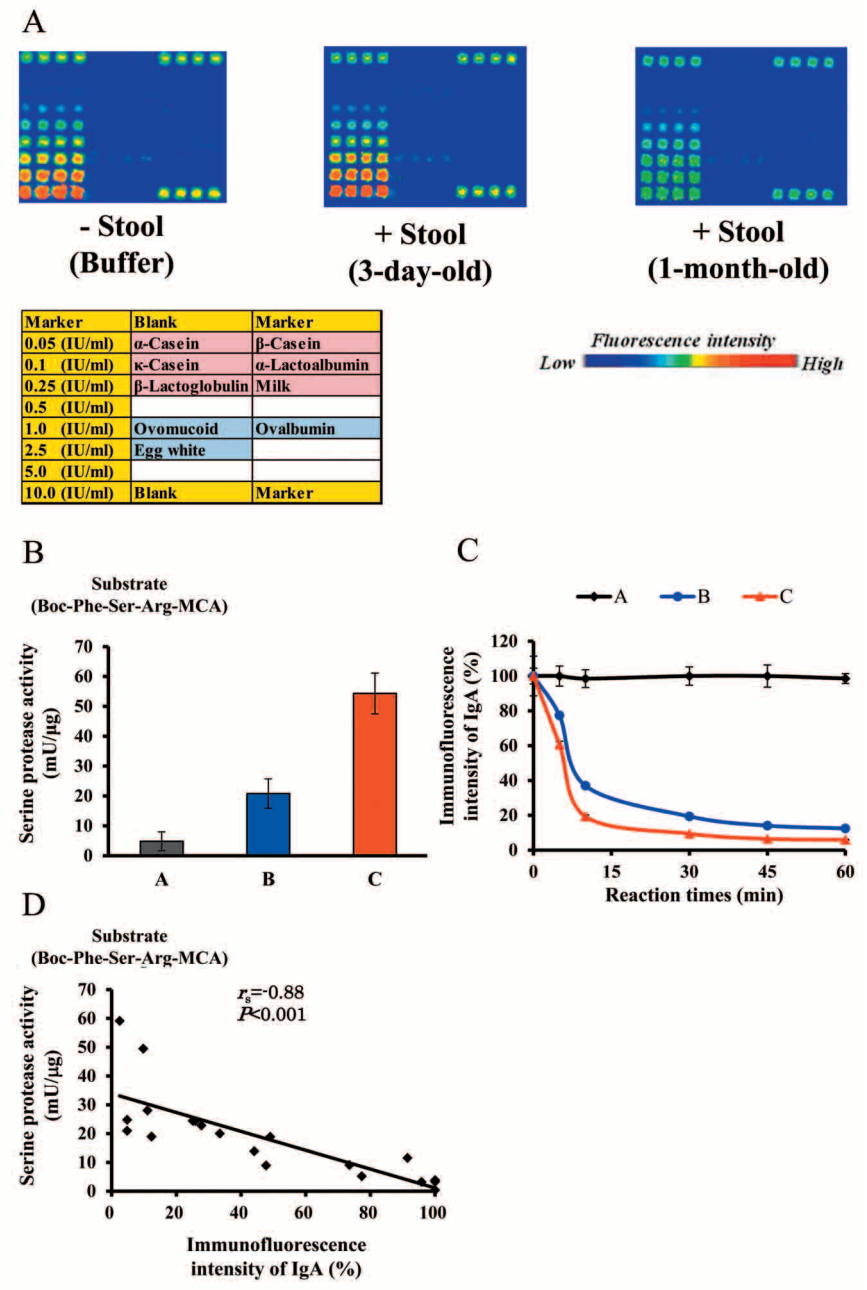

Fig. 1. Reduction of immunofluorescence intensities of IgA by protease(s) in stool of neonates and infants.
A : Rainbow displays of fluorescence intensities of SIgA and IgA on the protein chip after reaction with stool extracts (0.25 mg/mL) of 3-day-old neonate and 1-month-old infant. B : Serine protease activity (mU/μg protein) in stool extracts of 1-month-old infants (n=20) was measured and divided into group A (activities below 10 mU/μg protein, n=7), group B (activities between 10 and 30 mU/μg protein, n=11) and group C (activities above 31 mU/μg protein, n=2). Data represent the mean±SD of the number of stool samples in each group. C : Time courses of percent reduction in immunofluorescence signals of standard IgA were analyzed after incubation with each representative stool extract in group A (0.4 mU/μg protein), B (28.1 mU/μg protein) and C (59.1 mU/μg protein). The immunofluorescence intensity of IgA at 0 time was set at 100%. Data represent the mean±SD of the tetraplicates spotted standard IgA. D : Relationship between serine protease activity in stool and immunofluorescence of IgA. Stool extracts of 1-month-old infants (n=20) were incubated with allergen chips for 10 min and the percent reduction of immunofluorescence signals of standard IgA (10 IU/ml) was analyzed. Control immunofluorescence intensity of IgA in the absence of stool extract was set at 100%.

the immunofluorescence signals of WHO IgA standards were markedly reduced on the chip, compared with the control (no stool) and with stool of 3-day-old neonate. These data suggest that stool protease(s) degrades IgA standards, SIgA captured on allergens and/or allergens.

Since the major IgA-degrading protease(s) in stool of neonates, infants and toddlers is the Boc-Phe-Ser-Arg-MCA hydrolyzing trypsin-type serine protease(s) (*see below*), we measured the activities of trypsin-type serine proteases in 20 stool samples, prepared separately from 1-month-old infants, and divided into three groups : group A, (activities below 10 mU/μg protein), group B (activities between 10 and 30 mU/μg protein) and group C (activities above 31 mU/μg protein) (Fig. 1B). Fig. 1C shows the time course of reduction of immunofluorescence intensity of standard IgA (10 IU/mL) in the presence of representative stool samples from each group. While the fluorescence intensity of IgA in sample A did not change throughout the 60-min incubation at 37°C, the intensities in samples B and C rapidly and markedly decreased within 10-min incubation and remained low for up to 60 min. Fig. 1D shows the negative and significant correlation between stool serine protease activity and IgA fluorescence intensity after 10-min incubation (r_s=-0.88 ; p< 0.001).

Effects of protease inhibitors on stool-induced reduction of IgA immunofluorescence intensity

In the next step, we used various protease inhibitors to determine the exact protease responsible for the reduction of IgA immunofluorescence intensity induced by stools of 1-month-old infants. In the absence of protease inhibitors, stool reduced IgA fluorescence to about 10-25% of that of the control (no stool). Among the protease inhibitors tested, PMSF at 1 mM almost completely inhibited the reduction by tool extracts (0.25 mg protein/ml) whereas no significant inhibitory effects were detected by other inhibitors (Fig. 2A). PMSF is known to inhibit the activities of serine proteases, such as trypsin, chymotrypsin and thrombin, and cysteine protease papain but not other cysteine proteases and metalloproteases (29). The inhibitory effects of PMSF on the reduction of IgA immunofluorescence were dose-dependent, and concentrations of 0.5 mM and higher resulted in complete suppression (Fig. 2B). Thereafter we used 1 mM PMSF for the stool extracts at protein concentration of 0.25 mg/ml and 10 mM PMSF for those at protein concentration of 2.5 mg/ml.

We also tested the effects of various protease inhibitors on reduction of IgA immunofluorescence intensity induced by stools of 6-7-months-old infants and 1-3-years-old toddlers. These stool extracts also reduced IgA immunofluorescence, and PMSF did not result in complete inhibition (Fig. 3A and B). Instead, the stool-induced reduction was completely abrogated by a protease inhibitor mixture containing 1 mM PMSF, 0.1 mM leupeptin, 0.1 mM bestatin and 0.1 mM E-64c. To determine the effect of inhibitors other than PMSF on the reduction, two stool samples with incomplete restoration of the immunofluorescence intensity of IgA with PMSF in experiments A and B were selected and the effects of inhibitor combination with 1 mM PMSF were analyzed. Among the tested combinations, 1 mM PMSF plus 0.1 mM leupeptin produced complete inhibition of the stool-induced reduction of IgA immunofluorescence (Fig. 3C).

Reduction of allergen immunoreactivity by stool protease(s)

The protease(s) in stool of neonates, infants and toddlers can degrade not only IgA and SIgA but also allergens immobilized on the chip. Accordingly, we analyzed the loss of allergen immunoreactivity by stool of 1-month-old infants after incubation with the allergen chip for 60 min at 37°C and analyzed the allergen immunoreactivity. Reduction of allergen immunoreactivity was monitored by serum IgA of patients with egg and milk allergy. The

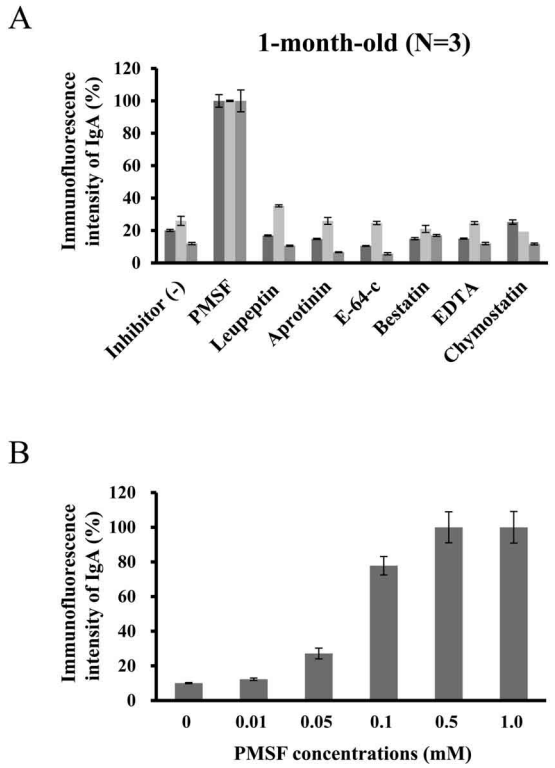

Fig. 2. Effects of protease inhibitors on reduction of immunofluorescence intensity of IgA induced by stool of 1-month-old infants.
A : Stools extracts (0.25 mg/ml) from 1-month-old infants (n=3) incubated with or without protease inhibitors (1 mM PMSF, 0.1 mM leupeptin, 0.1 mM aprotinin, 0.1 mM E-64c, 0.1 mM bestatin, 1 mM EDTA and 0.1 mM chymostatin) in PBS containing 5% DMSO, 0.3 M KCl and 0.05% Tween 20, were incubated with allergen chips for 60 min at 37°C and the immunofluorescence intensities of standard IgA (10 IU/ml) were analyzed. The immunofluorescence intensity of IgA in the absence of stool extract was set as 100%. Data represent the mean±SD of the tetraplicates spotted standard IgA. B : Effects of various doses of PMSF on the immunofluorescence intensities of IgA (10 IU/ml). One stool sample with the lowest immunofluorescence intensity without inhibitor in experiment A at 12.0% was selected, incubated with various doses of PMSF for 60 min at 37°C and then restoration of the immunofluorescence intensities of IgA on the chip was analyzed. The intensity of IgA in the absence of stool extract was set as 100%. Data represent the mean±SD of the tetraplicates spotted standard IgA.

immunofluorescence signals of allergens on the chip visualized by allergen-specific IgA from sera of allergic patients were markedly reduced by the stool extract and the reduced signals were almost completely restored by the addition of 1 mM PMSF to the stool extracts (Fig. 4A). The reduction of immunoreactivity and restoration were analyzed for each allergen. Since ovomucoid and β-lactoglobulin exhibit protease inhibitor activities, the immunofluorescence signal was not or only mildly reduced, respectively. However, the immunofluorescence signals of α-casein, β-casein, κ-casein, milk and egg white allergens were markedly reduced by the stool extracts but completely restored by 1 mM PMSF (Fig. 4B).

We also analyzed the reduction of immunoreactivity of SIgA by protease(s) in stool of 1-3-year-old toddlers (n=4). Fig. 5 shows representative results of loss of immunoreactivities against milk. The immunofluorescence signals of milk allergen-specific SIgA in the stool of four individuals were markedly reduced during 9-hr incubation in the absence of the protease inhibitors.

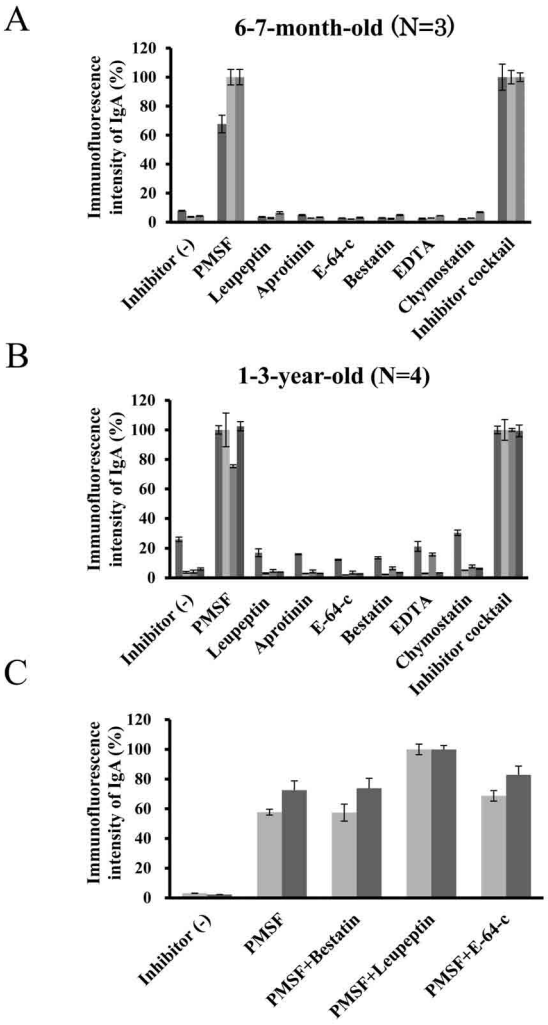

Fig. 3. Effects of protease inhibitors on the reduction of immunofluorescence intensity of IgA induced by stool extracts from 6-7-month-old infants and 1-3-year-old toddlers.
Stool extracts (0.25 mg/mL) from 6-7-month-old infants (n=3) (A) and 1-3-year-old toddlers (n=4) (B) with or without protease inhibitors, 1 mM PMSF, 0.1 mM leupeptin, 0.1 mM aprotinin, 0.1 mM E-64c, 0.1 mM bestatin, 1 mM EDTA, 0.1 mM chymostatin and inhibitor mixture described in the text, were incubated with allergen chips for 60 min at 37°C and the immunofluorescence intensities of IgA (10 IU/ml) were analyzed. The imunofluorescence intensity of IgA in the absence of stool samples was set as 100%. Data represent the mean±SD of the tetraplicates spotted standard IgA.
(C) Effects of PMSF combined with other inhibitors on immunofluorescence intensity of IgA. Two samples with incomplete restoration of the immunofluorescence intensity of IgA with PMSF in experiments A and B were selected and the effects of inhibitor combination were further analyzed. The combinations used were : 1 mM PMSF plus 0.1 mM bestatin, 1 mM PMSF plus 0.1 mM leupeptin, 1 mM PMSF plus 0.1 mM E-64-c in PBS containing 5% DMSO, 0.3 M KCl and 0.05% Tween 20. Immunofluorescence intensities of IgA (10 IU/ml) were analyzed. The intensity of IgA in the absence of stool extract was set as 100%. Data represent the mean±SD of the tetraplicates spotted standard IgA.

Detection of allergen-specific SIgA in stool of neonates and infants

Under the experimental conditions of protease inhibitors stated above, which protect against the degradation of allergens, IgA and SIgA on the chip, we measured allergen-specific SIgA in stool samples from 3-day-old neonate and 1-month-old infant on the chip with protease inhibitor cocktail that contained 10 mM PMSF, 0.1 mM leupeptin and other protease inhibitors. Immunofluorescence signals of allergen-specific SIgA of egg white, ovalbumin, α-casein, β-casein, κ-casein and milk were detected using stool extracts (2.5 mg protein/ml) of 1-month-old infant (Fig. 6). However, the stool extract of 3-day-old neonate did not show such immunofluorescence signals probably due to the lack of SIgA at that age (30).

DISCUSSION

Analysis of the levels of allergen-specific immunoglobulins, cytokines and allergens in the gut mucosa is important to understand the status of gastrointestinal maturation and mucosal immunity, particularly in patients with gastrointestinal food allergies. Food allergies in neonates, infants and young children have increased significantly in the past decades but there was no precise report of the methods of stool immunoglobulin analysis.

In the present study, we measured allergen-specific SIgA in stools of neonates, infants and toddlers using a new sensitive DCP allergen-microarray that has been described recently for measurement of various immunoglobulin parameters in serum and saliva (19-23). We found that immunofluorescence signals of allergens on the chip, allergen-specific SIgA in stool and standard IgA immobilized on the chip were markedly decreased by PMSF-sensitive and partly leupeptin-sensitive serine protease(s) in stool. These results suggest that stool protease(s) degrades not only immunoglobulin IgA and SIgA but also various allergens, such as α-casein, β-casein, κ-casein, α-lactoalbumin, β-lactoglobulin, milk and egg white on the chip, except ovomucoid which has an activity of serine protease inhibitor (31).

The activities of various protease(s) in stool of neonates, infants and toddlers and the reduction of immunofluorescence signals of SIgA and IgA by stool protease(s) were to a large extent inhibited by PMSF and in part by leupeptin, but not by the other inhibitors tested in this study. The inhibitor spectrum indicates that protease(s) present in stool that degrade IgA, SIgA and allergens may be exogenous proteases, such as IgA_1, IgA_2, SIgA or IgG proteases in *Escherichia coli* (17, 18) and other microbiota, rather than being leupeptin- and chymostatin-sentitive pancreatic proteases, trypsin and chymotrypsin, respectively. In this regard, reduced IgA immunofluorescence signals were not detected in the stool of 3-day-old neonates probably because of immaturity (30). On the other hand, allergen-specific SIgA detected in the stool of 1-month-old infants most likely originated from breast-fed milk from the mother, which immunoreacted with milk and egg allergens. However, we cannot completely exclude the possibility that these SIgA originated in the infant gut (Fig. 6).

The intestinal microbial abundance, diversity, and composition change rapidly after birth by clinical, cultural, and environmental factors, such as mode of delivery, diet, environment, family environment, diseases, and therapies used (32). In fact, our results showed different spectrum of protease inhibitors in the stool of infants older than 6 months of age relative to that of neonates, which could explain the need for additional inhibitors, such as leupeptin, with PMSF to produce complete inhibition of stool protease(s) of infants during growth and maturation.

In the present study, we described a new method for measurement of allergen-specific SIgA in stool of neonates, infants and toddlers using a sensitive DCP allergen microarray in the presence of inhibitor cocktail, particularly with PMSF and leupeptin. The inhibitor cocktail may be useful not only for the analysis of allergen-specific immunoglobulin levels but also various cytokines, in studies

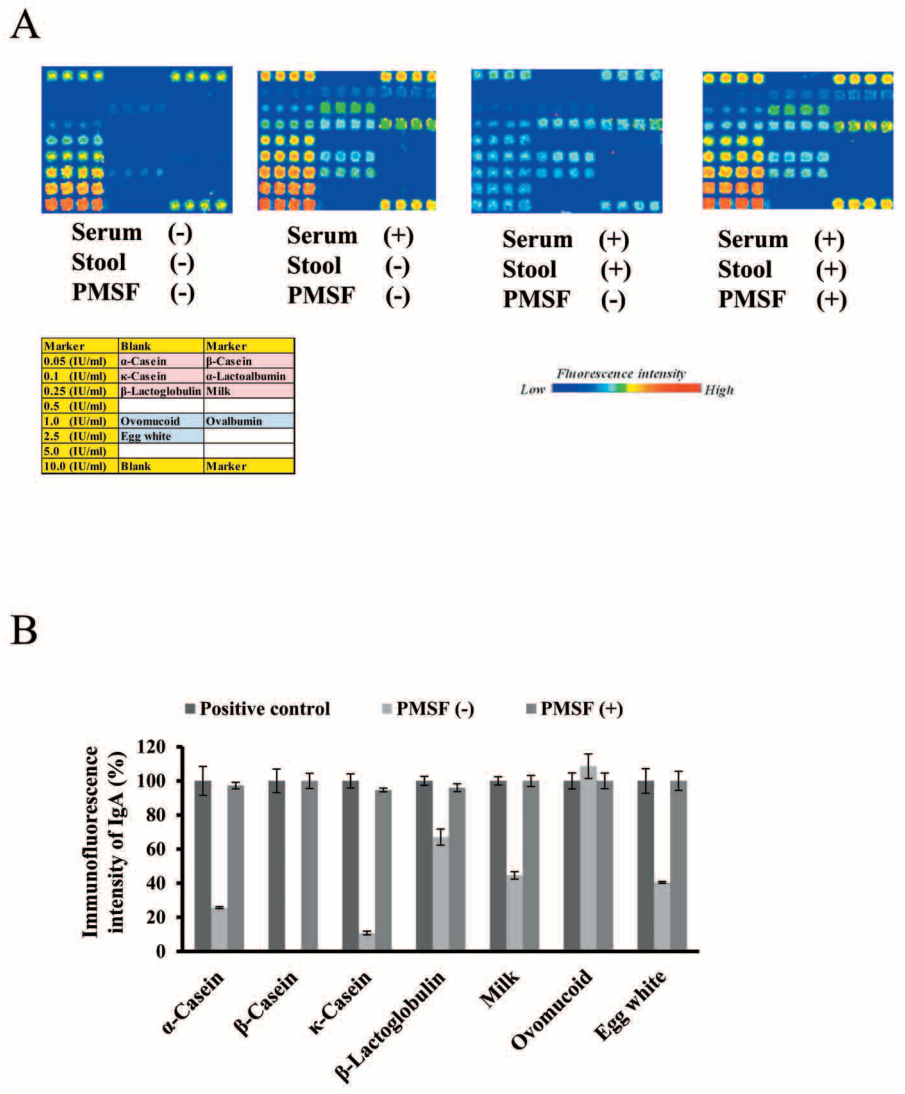

Fig. 4. Reduction of allergen immunoreactivity by stool protease(s) on a chip.
A : Allergen immunoreactivity was analyzed after incubation of immobilized allergen on the chips with stool of 1-month-old infant in the presence and absence of 1 mM PMSF for 60 min at 37°C. Reduction of allergen immunoreactivity was monitored using serum IgA from patients with known egg and milk allergy. B : Reduction of allergen immunoreactivity of each allergen by stool protease(s) and its restoration by 1 mM PMSF in stool extracts. Immunofluorescence intensity of allergen immunoreactivity was monitored as described in A. The intensity of each antigen measured in the absence of stool extract was taken as 100% (positive control). Data represent the mean±SD of the tetraplicates spotted standard IgA.

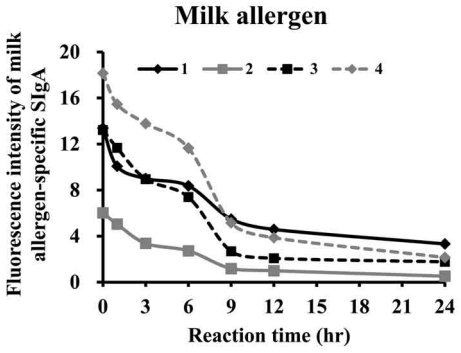

Fig. 5. Reduction of immunoreactivity of SIgA by protease(s) in stool of 1-3-year-old toddlers.
Stool extracts (2.5 mg/ml) free of protease inhibitors from 1-3-year-old toddlers (n=4) were incubated for various time intervals up to 24 hr at 37°C and then SIgA in the stool extracts were reacted with allergens on the chip for 60 min at 37°C. Representative data of SIgA immunoreactivity against milk as monitored by fluorescence intensity after reaction.

designed to analyze the pathogenesis of gastrointestinal food allergies in neonates, infants and toddlers.

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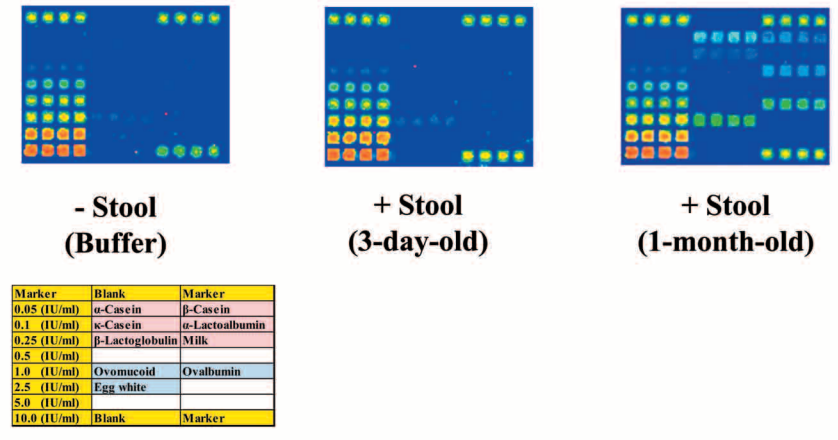

Fig. 6. Detection of allergen-specific SIgA in stool of neonate and 1-month-old infant.
Stool extracts (2.5 mg/mL) from 3-day-old neonate and 1-month-old infant in inhibitor cocktail (10 mM PMSF, 0.1 mM leupeptin, 0.1 mM bestatin and 0.1 mM E-64c) were incubated with allergen chips for 60 min at 37°C and then the fluorescence intensities of SIgA captured on each allergen were displayed as rainbow image.

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