Capsule Summary

Evidence for intrauterine sensitization of allergen-specific IgE in cord blood analyzed by a highly-sensitive new allergen diagnosis microarray.

INTRODUCTION

Newborns sometimes show measurable amounts of IgE antibodies in cord blood (CB) and a relatively high level of total IgE is often regarded a prenatal risk factor for atopic propensity in the newborn.[1-5] The latter conclusion is supported by the detection of allergen-specific IgE[6,7] and allergen-specific T-cell memory[8-10] in CB and suggests that primary sensitization can occur transplacentally *in utero*. However, the timing of allergen sensitization is still controversial, with conflicting evidence suggesting transplacental priming[6] versus postnatal priming[11, 12].

These conflicting conclusions could be due to the following background including the methods of analysis: (i) High probability of maternal blood (MB) contamination during cord blood sampling or through small placental bleeding during late pregnancy or delivery. (ii) Low sensitivity detection of allergen-specific IgE levels and allergen-specific IgE profiles against various food and inhalant allergens in CB. Since the majority of total IgE in CB is nonspecific IgE, generally much higher than allergen-specific IgE levels[11,13,14], detection of allergen-specific IgE and its profiles of CB are difficult; highlighting the need for the development of new highly-sensitive methods for the detection of allergen-specific antibodies. In a recent study[15], we described a new microarray technique of high-density antigen immobilization using the carboxylated arms on the surface of a diamond-like carbon (DLC)-coated chip, which had higher sensitivity in detecting allergen-specific IgE, IgA, IgG and IgG4, compared with the UniCAP system and allergen-specific immunoglobulins profiles against various food and inhalant allergens.

The present study is an extension to our previous study[15] and was designed to further examine the utility of the new method. Specifically, we used the DLC chip to detect allergen-specific IgE, IgA, IgG and IgG4 and determine the allergen profiling patterns in carefully-sampled CB to avoid MB contamination. The new technique identified

allergen-specific IgE in CB, which were of fetal origin. The results allowed analysis of the mechanism of allergen sensitization in the fetus and maternofetal transfer of immunoglobulins.

METHODS

Subjects

The study included 92 healthy paired pregnant women and their newborns recruited at Kawatetu-Chiba hospital, Chiba University hospital and Health Insurance Naruto hospital from January 2007 to May 2008 in Japan. At birth, CB was collected by needle puncture of the umbilical vein after careful cleaning the umbilical cord to avoid MB contamination. Neonatal blood (NB) was obtained at the time of birth by Contact-Activated Lancets low flow (BD Microtainer®) and venous MB was obtained at 4 to 5 days after delivery. Blood samples were then centrifuged at 150 × *g* for 10 min to prepare serum. Serum was frozen at -30°C until analysis. All subjects provided written informed consent to participate in this study. This study is approved by the ethics committees of the Graduate School of Medicine, Chiba University and Tokushima University hospital.

Allergen chip assay

Allergen-specific IgE, IgA, IgG and IgG4 levels were measured in serum by the allergen diagnosis DLC chip as described in detail previously.[15] Briefly, carboxylated DLC film-coated glass slide (Gene slide) was purchased from Toyo Kohan Co. (Tokyo, Japan). Natural allergens, *Dermatophagoides farinae* (Df) and *Dermatophagoides pteronyssinus* (Dp) were purchased from Allergon (Ängelholm, Sweden). Purified single allergens as molecular allergens, such as ovomucoid, ovalbumin, conalbumin, α-casein, β-casein, and

112 β-lactoalbumin, were from Sigma-Aldrich (St. Louis, MO). Japanese cedar was from Cosmo
113 Bio Co. (Tokyo) and house dust from GREER (Lenoir, NC). Human serum IgE (75/502), IgG,
114 IgA and IgM (67/086) used as internal standards on chip were from the National Institute for
115 Biological Standards and Control (Hertfordshire, England).

116 After activation of carboxylated DLC slides and fabrication of allergen microarray,
117 the individual arrays were incubated with 20 μL of 1:2 to 1:50 diluted serum as the primary
118 antibody, then reacted with a HiLyte Fluor™ 555 (Dojindo Molecular Technologies, Inc,
119 Kumamoto, Japan) labeled secondary antibody against each human IgE, IgA, IgG and IgG4,
120 and the resulting images were analyzed as described previously.[15] On each allergen chip,
121 various concentrations of human IgE, IgG or IgA were spotted as internal standards. From the
122 cubic equation of IgE, IgG or IgA standard concentrations, the amounts of allergen-specific
123 antibodies bound to allergen on the chips were calculated and expressed as Binding Unit
124 (BU). The BUs of IgE, IgA, IgG and IgG4 were reported as BUe, BUa, BUg and BUg4,
125 respectively. The detection limit of allergen-specific IgE against various natural and
126 molecular allergens in serum in the DLC chip was 10 BUe/mL, which corresponds to about
127 0.07 IU/mL of the UniCAP system, indicating about 4 to 8 times higher sensitivity for DLC
128 chip than the UniCAP system. The UniCAP system has a limit for IgE detection of 0.35
129 IU/mL[6,16]. The detection limits for allergen-specific IgA, IgG and IgG4 were 0.25 BUa, 2.50
130 BUg and 0.53 BUg4, respectively.

131 We compared the sensitivity of the DLC chip to that of the UniCAP system for
132 allergen-specific IgE in CB, which contains a relatively high level of nonspecific IgE[11, 13, 14]
133 (TABLE I). The UniCAP system did not detect allergen-specific IgE in all CB samples
134 analyzed in our experiments, even in samples of fluorescence units (BUe/mL) of more than
135 18 to 22 times the detection limit (10 BUe/mL) on the DLC chip. However, the difference in
136 the sensitivity between the DLC chip and the UniCAP system using MB samples was

equivalent to that in allergic patients[15] described above.

Total IgA assay

Total IgA concentration was determined using an enzyme-linked immunosorbent assay (ELISA) kit (Bethyl Laboratories, Montgomery, TX) according to the protocol provided by the manufacturer. The chromogen produced was measured at absorbance of 450 nm using a SpectraMax Plus384 autoreader (Molecular Devices Corp., Sunnyvale, CA).

Statistical analysis

Statistical analysis was conducted using The Statistical Package for Social Sciences (version18.0; SPSS Inc, Chicago, IL). Most data sets showed skewed distribution and thus Spearman's rank correlation test was used to assess the relationship between the different samples. A P value $\leq .05$ was considered significant.

RESULTS

Allergen-specific serum IgE, IgA, IgG and IgG4 levels and their profiles in CB, NB and MB

Allergen-specific IgE, IgA, IgG and IgG4 levels in CB, NB and MB and their profiles were analyzed by the allergen diagnosis DLC chips. The DLC chip detected more than one allergen-specific IgE of the tested 11 allergens in 83.7% of CB (n = 92). Figure 1 shows allergen-specific IgE, IgA, IgG and IgG4 profiles in representative paired CB, NB and MB samples. MB contained high-reactive IgE levels against inhalant allergens, Dp and Df, and moderate-reactive IgE levels against food allergens, milk, α-casein, β-casein and also inhalant allergens, cedar pollen and house dust. CB, however, did not contain any reactive

IgE against inhalant allergens, Dp, Df, cedar pollen and house dust, but had moderate reactive IgE levels against food allergens, milk, α-casein, β-casein, as well as ovomucoid. Although MB contained various allergen-specific IgA, CB did not show any reactive IgA. Almost identical levels of allergen-specific IgG and IgG4 to each allergen and similar profile patterns were observed among MB, NB and CB. The difference in the allergen-specific profiles of IgA and IgE between MB and CB or NB indicates no MB contamination in the paired CB samples and suggests that allergen-specific IgE in CB is derived from the fetus. The almost identical allergen-reactive profiles of IgG and IgG4 among CB, NB and MB support the established finding of maternofetal transfer of IgG.[17] Similar findings were observed in the other 91 paired CB and MB samples.

To evaluate the cross-reactivity of the antigen-IgE antibody reaction on the highly-sensitive DLC chip, serum was pre-incubated with each allergen for 2 hours at 37°C followed by allergen-specific IgE detection on the chip (Fig. 2). Each allergen selectively and almost completely adsorbed allergen-specific IgE antibodies without any interference or cross-reactivity by other antigen-antibody reactions. These results indicate that allergen-specific IgE was selectively detected on the DLC chip.

Total IgA levels in CB and MB

Since total IgA level in CB is generally used as an indicator of transfer of MB[18], we measured total IgA levels in CB and MB by ELISA. Total IgA levels in all CB were within the minimal levels between 1.2 and 19.4 μg/mL (Fig. 3) and no allergen-specific IgA was detected (Fig. 1 and TABLE II). In contrast, total IgA levels in MB were between 0.8 and 3.5 mg/mL. Therefore, the total IgA levels in MB did not correlate with those in CB. These results indicate that MB contamination is below the detection level in CB collected by careful needle puncture of the umbilical vein.

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188 **Allergen-specific IgE, IgA, IgG and IgG4 levels in CB and MB**

189 Allergen-specific IgE, IgA, IgG and IgG4 levels were analyzed in 92 paired CB and
190 MB samples. The proportion of CB samples positive for allergen-specific IgE against each
191 food allergen (using a cutoff value of 10 BUe) ranged from 6.5 to 69.6%, with the highest
192 proportion for ovomucoid, while the proportion of samples positive for each inhalant allergen
193 ranged between 6.5 and 28.3% (TABLE II). The proportions of MB samples positive for
194 allergen-specific IgE against each food allergen were almost similar to those of CB. In
195 contrast, the proportions of MB samples positive for allergen-specific IgE against inhalant
196 allergens were considerably higher (between 72.8 and 84.8%) than those of CB. Specifically,
197 the proportion of CB samples positive for allergen-specific IgE with IgE-positive MB only
198 against each food allergen was high and the mean value was 86.4%. The mean BU ratio for
199 CB/MB in IgE-positive subjects only against the food allergens (i.e., CB BUe/MB BUe,
200 TABLE II) was 1.24±0.60. In contrast, the proportion of CB samples positive for
201 allergen-specific IgE in the IgE-positive MB subjects only against the inhalant allergens was
202 lower (range, 9.0-33.8%), with a mean value of 20.7%. The mean BU ratio for CB/MB in the
203 IgE-positive subjects only against inhalant allergens (i.e., CB BUe/MB BUe, TABLE II) was
204 0.54±0.50. These results suggest a potentially greater sensitization against food allergens than
205 inhalant allergens *in utero*.

206 The levels of allergen-specific IgA against the allergens tested in CB were below the
207 detection limits but the proportion of MB samples positive for allergen-specific IgA against
208 food and inhalant allergens ranged between 7.6 and 77.2%, with the highest value against
209 milk (TABLE II). A fairly similar or identical trend was noted in CB and MB for
210 allergen-specific IgG against food and inhalant allergens (TABLE III). The mean proportions
211 of CB samples positive for allergen-specific IgG against food and inhalant allergens in

212 subjects with IgG-positive MB only were 91.2% and 87.3%, respectively, and the mean BU
213 ratios of CB/BM in the IgG-positive subjects only (CB BUg/MB BUg, TABLE III) against
214 food and inhalant allergens were 1.08±0.38 and 0.87±0.24, respectively. The similar
215 proportions of CB and MB samples and BU ratios around 1.0 indicate materno-fetal transfer
216 of allergen-specific IgG.

217 Although the mean proportion of allergen-specific IgG4-positive CB in the
218 IgG4-positive MB subjects only against food allergens was 96.0%, those against inhalant
219 allergens were below the detection levels in the samples tested (TABLE III). The mean BU
220 ratio between IgG4-positive CB and IgG4-positive MB subjects only (CB BUg4/MB BUg4)
221 against food allergens was 0.99±0.39. The results add support to the notion of materno-fetal
222 transfer of allergen-specific IgG4.

223

224 **Correlation of allergen-specific IgE, IgA, IgG and IgG4 between CB and MB**

225 The results of correlation analysis of allergen-specific IgE, IgA, IgG and IgG4 levels
226 (BU) between paired CB and MB samples (n = 92) determined by the DLC chip are shown in
227 TABLE IV. There were strong correlations for allergen-specific IgE levels against food
228 allergens between CB and MB with considerably high correlation coefficients (range,
229 0.53-0.94, $P<.001$), with the exception of weak correlation for α-lactoalbumin at 0.37
230 ($P<.001$). In contrast, the correlation coefficients for inhalant allergens were low (range,
231 0.01-0.30). In addition, there were strong correlations for allergen-specific IgE against crude
232 allergens between CB and MB in the IgE-positive MB subjects only (TABLE II), such as
233 milk, egg white and cedar pollen, with high correlation coefficients of 0.87 ($P<.001$), 0.81
234 ($P<.001$) and 0.83 ($P<.042$), respectively. The correlation profiles of allergen-specific IgE
235 against various food and inhalant allergens for CB and MB are depicted in Fig. 4.

236 There were also significant and strong correlations for allergen-specific IgG against

237 food and inhalant allergens between CB and MB (r_s>0.74, P<.001), except allergen-specific
238 IgG against cedar pollen and α-lactoalbumin (r_s, not available) and against β-lactoglobulin
239 (r_s=0.58, P<.001) (TABLE IV). There were also strong correlations for allergen-specific
240 IgG4 against food allergens between CB and MB (r_s >0.85, P<.001), although that for
241 allergen-specific IgG4 against α-lactoalbumin was weaker (r_s = 0.68, P<.001). The
242 correlation coefficients between CB and MB were not available for allergen-specific IgA
243 against the allergens tested (TABLE II). Furthermore, the correlation coefficients for
244 allergen-specific IgG4 against inhalant allergens were not available because allergen-specific
245 BUg4 values were below the detection levels (TABLE III and IV). The correlation profiles
246 for allergen-specific IgG and IgG4 against each allergen between CB and MB are shown in
247 Supplementary figures 1 and 2.
248
249 **DISCUSSION**
250 We developed recently a new allergen diagnosis microarray with high sensitivity
251 using DLC-coated chips for profiling allergen-specific IgE, IgA, IgG and IgG4 against food
252 and inhalant allergens. The DLC chip allows lowering the limit of detection of
253 allergen-specific IgE in the UniCAP system to further dilution at 4–8-fold for each allergen[15]
254 and the detection limit of allergen-specific IgE in the DLC chip was about 5 times higher
255 sensitivity than the UniCAP system in MB and serum of allergic patients. The present study
256 demonstrated a larger difference in the detection sensitivity between the DLC chip and the
257 UniCAP system in CB than in MB (TABLE I). The reason for the larger difference is not
258 clear at this stage, but a relatively high level of nonspecific IgE in CB may disturb the
259 detection of allergen-specific IgE on the UniCAP system but not on the DLC chip system.
260 The latter immobilizes extremely high-density antigens on the surface of the diamond-like
261 carbon-coated chip[15] and maintains antigen-antibody reactivity even in the presence of high

levels of nonspecific IgE. Our highly sensitive allergen-specific IgE detection system is suitable for detection of low levels of allergen-specific IgE in CB compared with other previous methods.

The presence of IgE antibodies in CB has been analyzed extensively in the past 20 years since it is important in the design of allergy prevention strategies, particularly allergen avoidance during pregnancy.[1-5, 19-22] However, there is conflicting evidence on whether allergen-specific IgE in CB is a reflection of fetal immunity or the result of transfer of maternal IgE to the fetus. The controversy is probably related, at least in part, to the low sensitivity of the methods used for the detection of allergen-specific IgE in CB, and precise allergen-specific IgE profiling patterns against food and inhalant allergens are not available at present. Measurement of total IgE level in CB is not recommended for allergy risk screening.[6] Furthermore, CB sampling by means of needle puncture of the umbilical vein is essential to avoid MB contamination. To deal with these problems, we collected CB by needle puncture of the umbilical vein and analyzed allergen-specific IgE and other Igs both in CB/NB and MB using the newly developed highly-sensitive allergen diagnosis DLC chip.

The allergen diagnosis DLC chip detected allergen-specific IgE against more than one of the allergens tested in 83.7% of CB from infants analyzed. The rate of detection was higher than those reported previously[6,11], most likely due to the highly sensitive (TABLE I) and selective detection of allergen-specific IgE by the DLC chip (Fig. 2). The representative data of allergen-specific IgE profiling patterns of CB and NB (Fig. 1) showed characteristic patterns which were not identical to that in the paired MB. These results indicated lack of contamination of MB in CB and that IgE in CB is a product of the fetus. If IgE in CB is derived from MB through maternofetal transfer[11], the allergen-specific profiling pattern of the CB should be similar or identical to that of the MB. The results of the DLC chip of no perfect match of the allergen profiles of the CB and MB in the paired 92 samples tested support the

conclusion that the allergen-specific IgE identified in CB are of fetal origin.

It has been reported that IgA does not cross the placental barrier and is not produced *in utero* in significant amounts[23]. In contrast, maternal IgG are transferred to the fetus across the placenta by a specific receptor-mediated mechanism.[24,25] The total IgA levels in CB are commonly used to estimate MB contamination and levels greater than 50 μg/L indicate MB contamination.[11,18] In the present study, the total IgA levels in CB of all our samples were <50 μg/mL (range, 1.2-19.4 μg/mL), indicating no or minimal MB contamination. The reliability of the data from the DLC chip was also confirmed by the allergen-specific profiles of IgA, IgG and IgG4 (Fig 1): the obvious mismatch of the allergen-specific IgA profile of CB and MB supports no maternofetal transfer of IgA. On the other hand, the similar allergen-specific IgG and IgG4 profiles in CB and MB provide support to the maternofetal transfer of IgG.

The mean proportion of allergen-specific IgE-positive CB with IgE-positive MB against food allergens was 86.4%, which was about 4 times that with IgE-positive MB against inhalant allergens (20.7%), and their mean allergen-specific IgE BU ratios (CB BUe/MB BUe) for food and inhalant allergens were 1.24 and 0.54, respectively (TABLE II). These results may provide interpretation for the findings shown in Fig. 4 and TABLE IV of higher levels of allergen-specific IgE (BUe) in CB against food allergens than those against inhalant allergens, and strong and significant correlations between CB and MB for food allergen-specific IgE levels, but weaker correlations for inhalant allergen-specific IgE. These data suggest that maternofetal transfer of food allergens is more frequent and easier than inhalant allergens, although previous studies showed crossing of food and inhalant allergens through the placenta in *ex vivo* models.[26,27]

The mechanisms of maternofetal transfer of allergens have been discussed extensively, including fetus allergen-uptake[6] of allergen-IgG complexes through the amniotic fluid by aspiration or permeation through the fetal skin[22,28] and through active transplacental

312 transport[29]. Therefore, the presence of allergen-specific IgG levels in MB and CB may
313 increase the risk of maternofetal allergen transfer and induction of allergen-specific IgE in
314 CB.[30] Furthermore, it has been shown that the fetal immune system can produce IgE
315 antibodies from week 11 of gestation[13], and thus maternofetal transfer of allergen may trigger
316 allergen-specific IgE production *in utero*. Once these food and inhalant allergens are
317 transferred across the barrier, they may induce allergic sensitization *in utero* under the
318 influence of maternal immune conditions.

319 In our experiments, however, the mean allergen-specific IgG BU ratios (CB BUg/MB
320 BUg) for food and inhalant allergens were not significantly different at 1.08 and 0.87,
321 respectively (TABLE III) and allergen-specific IgG levels do not necessarily explain the
322 difference in the levels of IgE (BUe) in CB against food and inhalant allergens. At present,
323 the reasons for the difference in the proportion of allergen-specific IgE-positive CB and the
324 levels of IgE against food and inhalant allergens are not clear. To analyze this difference,
325 further measurements should be conducted of food and inhalant allergen levels in CB and
326 maternal circulation at the time of delivery.

327 Previous studies reported the presence of low (undetectable) levels of
328 allergen-specific IgE in infant blood during the breast-feeding period at 6 months of age,
329 compared with detectable levels of allergen-specific IgE in CB of some infants.[11,31] This
330 observation might be due to the separation after birth from the source of allergens (i.e.,
331 amniotic fluid and transplacental transport) and also from the maternal immune system. The
332 findings of sequential appearance of first food-related and later in the preschool age of
333 inhalant allergen-related IgE despite constant environmental exposure to the inhalant
334 allergens by birth is a common knowledge. To study the mechanisms of age-dependent
335 changes in the allergic phenotypes, simultaneous measurements of antigen-specific IgE, IgA,
336 IgG1, IgG4 and IgG in serum, nasal secretion and saliva by the DLC chip as well as

measurements of cytokine levels in these samples might be helpful. The present study found allergen-specific IgG against food and inhalant allergens but no allergen-specific IgG4 against inhalant allergens in MB and CB. Further studies are also required on the relationship between allergen-specific IgE, IgG and IgG4 inductions in fetus and early infantile allergy against food and inhalant allergens.

Conclusions

Analysis using a highly-sensitive DLC microarray for allergens demonstrated differences in allergen-specific IgE profiles in 92 paired MB and CB/NB samples. The finding clearly indicates that IgE levels in CB reflect *in utero* sensitization.

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443 Clin Immunol 2008;121:646-51.

TABLE IV. Correlation of allergen-specific IgE, IgG and IgG4 BU between the 92 paired CB and MB.

Allergen	Correlation between CB and MB					
	IgE		IgG		IgG4	
	r_s	*P*	r_s	*P*	r_s	*P*
Food						
α-Casein	0.69	<.001	0.92	<.001	0.94	<.001
β-Casein	0.70	<.001	0.74	<.001	0.85	<.001
α-Lactoalbumin	0.37	<.001	NA		0.68	<.001
β-Lactoglobulin	0.59	<.001	0.58	<.001	0.97	<.001
Ovalbumin	0.56	<.001	0.93	<.001	0.96	<.001
Ovomucoid	0.94	<.001	0.94	<.001	0.97	<.001
Milk	0.60	<.001	0.91	<.001	0.95	<.001
Egg white	0.53	<.001	0.94	<.001	0.97	<.001
Inhalant						
Cedar pollen	0.30	.004	NA		NA	
Df	0.01	.906	0.83	<.001	NA	
Dp	0.19	.073	0.83	<.001	NA	

NA, Not available due to lack of positive cases.

469 Allergen-specific IgE levels in CB and MB (n = 92) depicted in BUe. The cutoff value was
470 10 BUe. (a) Milk, (b) α-Casein, (c) Ovomucoid, (d) Cedar pollen, (e) Dp and (f) Df.
471 Spearman's rank correlation test was used to assess the relation between the values of CB and
472 MB.

TABLE I. Comparison of assay sensitivity in detecting antigen-specific IgE in CB and MB against food allergens and inhalant allergens using the DLC chip system and UniCAP system.

Allergen	CB (1:1 dilution)		MB (1:1 dilution)	
	DLC chip (BUe/mL)	UniCAP (PRU)	DLC chip (BUe/mL)	UniCAP (PRU)
Food				
Egg white	30.35	ND#	77.41	0.545
	11.02	ND	23.65	ND
Ovomucoid	180.0	ND	134.8	0.960
	84.89	ND	64.66	ND
	13.30	ND	23.15	ND
Milk	221.4	ND	182.7	1.095
	30.90	ND	61.71	0.540
	18.05	ND	23.15	ND
Inhalant				
Cedar pollen	55.55	ND	90.98	0.960
	21.78	ND	32.20	ND
Df	54.01	ND	80.76	1.275
	47.38	ND	25.53	ND
Dp	63.04	ND	215.6	2.950
	26.48	ND	60.70	ND

CB serum (1:1 dilution) and MB serum (1:1 dilution) were used for measurement of allergen-specific IgE levels on the UniCAP system and the DLC chip.

ND# of UniCAP assay: <.35

Detection limit on the DLC chip: 10 BUe/mL