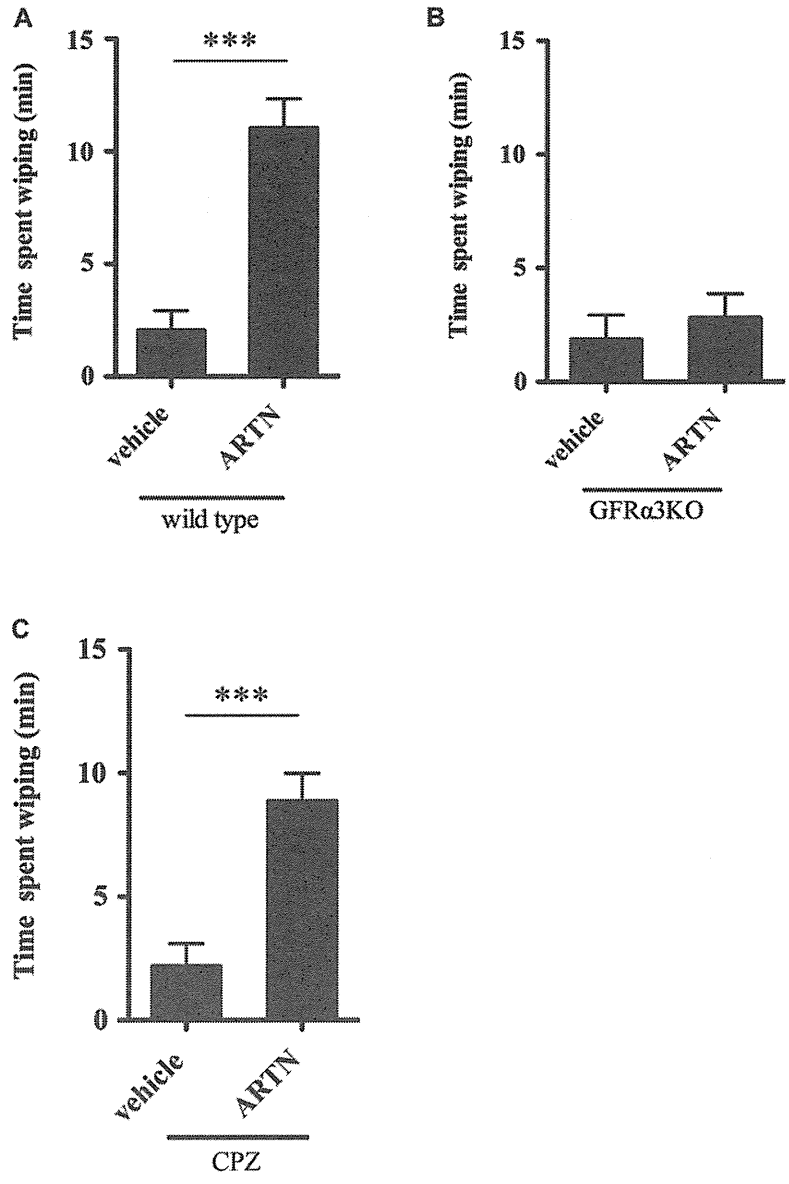

FIG 6. Artemin-injected mice showed abnormal behavior in warm conditions. **A,** Artemin-injected wild-type mice were videotaped, and the time spent wiping their cheek was measured. Representative of 2 independent studies (n = 3 in each experiment). **B,** Artemin-injected GFRα3KO mice were videotaped, and the time spent wiping their cheek was measured. Representative of 2 independent studies (n = 3 in each experiment). **C,** The impact of capsazepine (CPZ) on artemin-induced abnormal behavior was measured (n = 3). ***$P < .001$.

NGF, which is known to have an important effect on cells of both the nervous and immune systems,[33,34] is expressed at higher levels in chronic inflammatory disorders including AD.[35,36] Although the sources of NGF are mainly keratinocytes, mast cells, and skin cells, dermal fibroblasts also produce low levels of NGF under basal conditions, and NGF itself or TGF-β can enhance its production.[37] It has been reported that NGF and artemin play distinct and essential roles in the development of sympathic axons toward their final configurations.[18] Sympathetic neuron development requires signaling by chemoattractant artemin for migration and initial axon outgrowth. Once the nerve fibers reach their proper target, their survival and maintenance depend on target-derived NGF instead of artemin.[18] These findings suggest that coordinated expression of artemin and NGF is probably important in the sprouting and abnormal elongation of cutaneous nerve fibers, which is frequently observed in itchy allergic dermatitis. In this study, the expression of NGF and artemin mRNAs was differently regulated by the concentration of SP, indicating that the tissue concentration of SP might determine whether NGF or artemin plays a dominant role in disorganized skin innervation.

Dermal fibroblasts also expressed both GDNF and GFRα1, suggesting that autocrine secretion of neutrophic factors may regulate the homeostasis of skin including tissue remodeling and innervation. However, the neutralization of GDNF did not affect the proliferative activity of SH-SY5Y cells cultured with conditioned medium derived from SP-treated fibroblasts. At present, we have no data that address this conflicting finding regarding the function of GDNF.

Recently, Davis and coworkers[38] reported a phenotype of thermal hyperalgesia in transgenic mice that overexpress artemin in skin keratinocytes (K14-artemin Tg mice) and proposed that the phenotype is probably due to the upregulation of TRPV1 on cutaneous peripheral nerve fiber. As the complaint of intractable heat-provoked itch is frequently observed in patients with AD,[4] it may be that both artemin and TRPV1 are involved in this type of itch. Our findings confirm that artemin-treated mice show curious behavior similar to heat-provoked scratching. As the inhibition of TRPV1 with capsazepine administration did not affect the artemin-induced abnormal behavior, this phenotype is probably independent of TRPV1. Meanwhile, it was an unexpected outcome that artemin-injected mice rubbed their cheek in a warm environment but not the injection site. At present, we cannot explain the mechanism with concrete data, and take it as given that artemin might induce alloknesis throughout the whole body. Another interesting phenotype of K14-artemin Tg mice is the elongation of peripheral nerve fibers into the epidermis, which suggests a possible role for artemin in axon guidance.[38] In this study, we obtained data supporting this role for artemin, by confirming an effect of artemin on the elongation of the peripheral nerve fibers. We conclude that artemin has a considerable impact on both thermal susceptibility and innervation of skin.

As noted above, both prurigo nodularis accompanied by itch and psoriasis unaccompanied by itch displayed less intense staining for artemin than AD. Reduced intraepidermal nerve fiber density has been thought to be an indicator of subclinical cutaneous neuropathy,[39] which consistent with the reduced expression of artemin, an inducer of intraepidermal neurite outgrowth, was low in prurigo nodularis. Thus, the different results with the different types of lesions associated with itch indicate that altered artemin expression does not underlie itch in all skin disorders. Exploring the role of artemin in nummular eczema is a subject of future investigation. Our findings indicate that artemin may contribute to a novel mechanism for warmth-induced itch and that further investigation will yield a better understanding of the pathogenic involvement of SP in AD.

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Key messages

- Although warmth-evoked itch is a problem to be solved in AD, the underlying mechanism remains obscure.
- Artemin was induced by SP from dermal fibroblasts and accumulated in the dermis of AD-lesional skin.
- Artemin causes skin nerve fiber sprouting and thermohyperesthesia and developed warmth-evoked scratching behavior.

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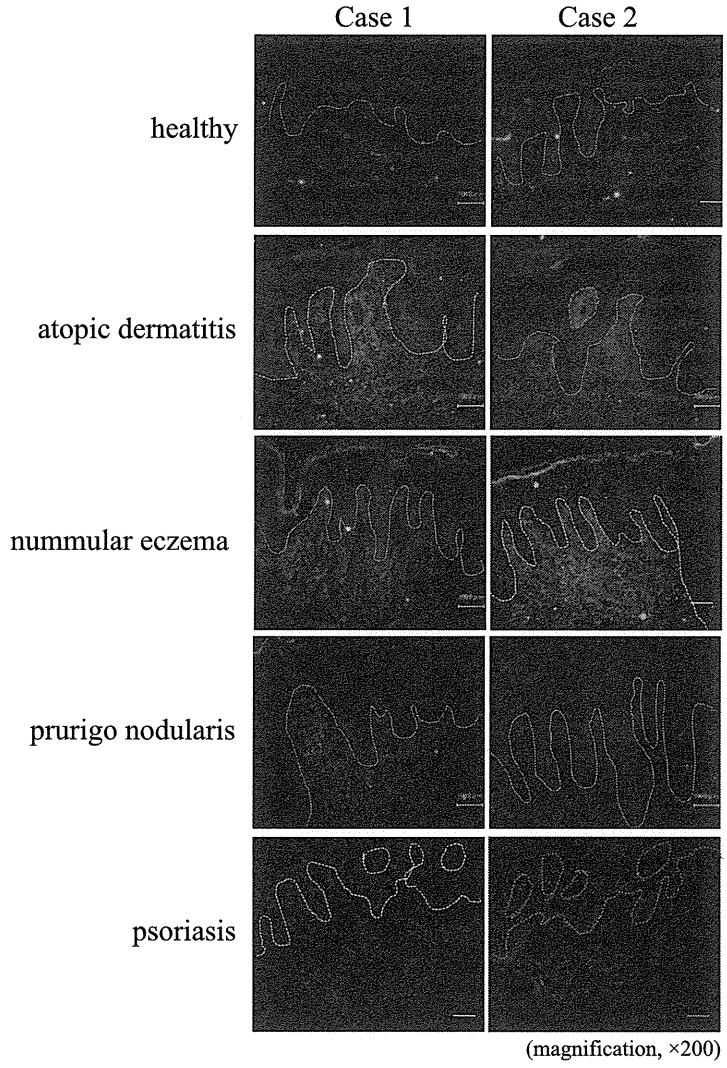

FIG E1. Immunohistochemical staining for artemin *(green)* was performed with healthy skin and 2 AD skin lesions (disease duration of case 1 and case 2 was about 1 week and 3 months, respectively), nummular eczema skin lesions (disease duration of case 1 and case 2 was about 1 month and 2 months, respectively), prurigo nodularis skin lesions (disease duration of case 1 and case 2 was about 1 year and about 6 months, respectively), and psoriasis skin lesions (disease duration of case 1 and case 2 was about 1 week and about 1 month, respectively). *Blue*: Hoechst 33342. *Dashed white lines* represent the epidermal-dermal junction. *Scale bar:* 100 μm.

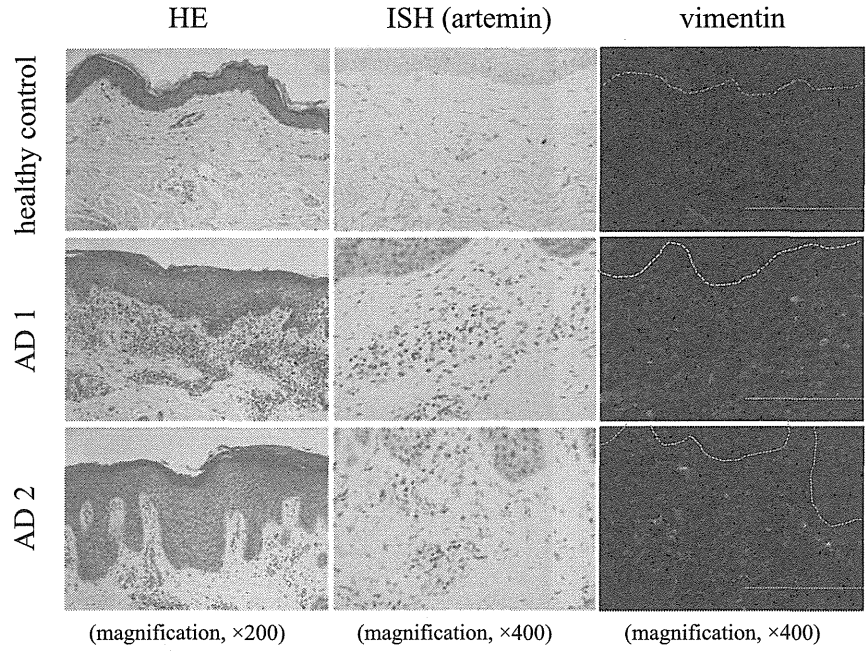

FIG E2. To compare the number of dermal fibroblasts, healthy skin and 2 AD skin lesions (AD1 and AD2), which were identical to the sample in ISH analysis (Fig 2, *D*), were costained for vimentin. Results of hematoxylin and eosin *(HE)* staining and ISH for artemin (*purple* indicates a positive signal) are shown alongside vimentin-stained images (*green*: vimentin, *blue*: Hoechst 33342). *Dashed white lines* in the vimentin-stained images represent the epidermal-dermal junction. *ISH, In situ* hybridization.

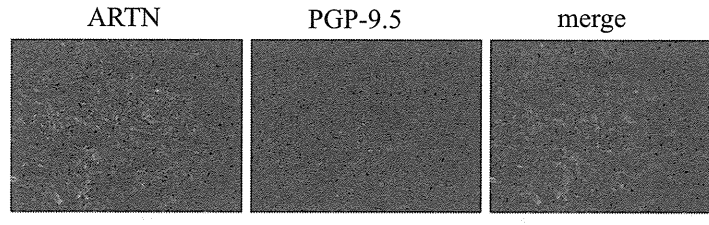

FIG E3. Skin innervation in AD lesional skin was examined by immunolabeling for PGP-9.5 and artemin. PGP-9.5–positive peripheral nerve fibers *(red)* showed massive sprouting in the area with artemin accumulation *(green)* (magnification ×400).

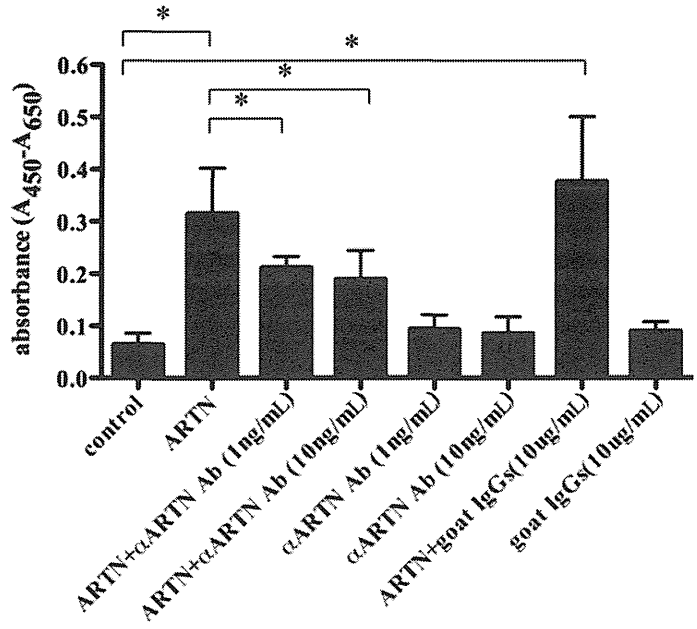

FIG E4. Artemin neutralization inhibited the rARTN-induced proliferation of SH-SY5Y cells, whereas an isotype matched control antibody did not. *ARTN,* Artemin; *AARTNAb,* artemin neutralizing antibody. *$P < .05$.

Intrauterine sensitization of allergen-specific IgE analyzed by a highly sensitive new allergen microarray

Norio Kamemura, MSc,[a] Hitomi Tada, MSc,[a] Naoki Shimojo, MD, PhD,[b] Yoshinori Morita, MD, PhD,[b] Yoichi Kohno, MD, PhD,[b] Takao Ichioka, MD,[c] Koichi Suzuki, MSc,[a] Kenji Kubota, MSc,[a] Mineyoshi Hiyoshi, PhD,[a] and Hiroshi Kido, MD, PhD[a] *Tokushima, Chiba, and Naruto, Japan*

Background: To design a rational allergy prevention program, it is important to determine whether allergic sensitization starts *in utero* under the maternal immune system.
Objective: To investigate the origin of allergen-specific IgE antibodies in cord blood (CB) and maternofetal transfer of immunoglobulins.
Methods: The levels of food and inhalant allergen-specific IgE, IgA, IgG, and IgG_4 antibodies in CB and maternal blood (MB) from 92 paired neonates and mothers were measured by using a novel allergen microarray of diamond-like-carbon–coated chip, with high-sensitivity detection of allergen-specific antibodies and allergen profiles.
Results: The levels of allergen-specific IgE antibodies against food and inhalant allergens and allergen profiles were identical in CB and newborn blood, but the levels and profiles, specifically against inhalant allergens, were different from those in MB. The level of allergen-specific IgA antibodies was below the detection levels in CB despite clear detection in MB. Therefore, contamination with MB in CB was excluded on the basis of extremely low levels of IgA antibodies in CB and the obvious mismatch of the allergen-specific IgE and IgA profiles between CB and MB. However, the levels of allergen-specific IgG and IgG_4 antibodies and their allergen profiles were almost identical in both MB and CB.
Conclusion: Allergen-specific levels of IgE and IgA antibodies and their allergen profiles analyzed by the diamond-like-carbon allergen chip indicate that IgE antibodies in CB are of fetal origin. Food-allergen specific IgE antibodies were detected more often than inhalant-allergen specific IgE antibodies in CB, the reason of which remains unclarified. (J Allergy Clin Immunol 2012;130:113-21.)

Key words: *Prenatal, allergen-specific IgE, IgA, IgG, and IgG_4, sensitization, cord blood, allergen chip*

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: (1) High probability of maternal blood (MB) contamination during CB sampling or through small placental bleeding during late pregnancy or delivery. (2) 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] the 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 IgG_4 compared with the UniCAP system and allergen-specific immunoglobulin 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 IgG_4 and determine the allergen profiling patterns in carefully sampled CB to avoid MB contamination. The new technique identified allergen-specific IgE antibodies 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

From [a]the Division of Enzyme Chemistry, Institute for Enzyme Research, University of Tokushima, Tokushima; [b]the Department of Pediatrics, Graduate School of Medicine, Chiba University, Chiba; and [c]the Health Insurance Naruto Hospital, Naruto.

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Corresponding author: Hiroshi Kido, MD, PhD, Division of Enzyme Chemistry, Institute for Enzyme Research, University of Tokushima, Kuramoto-cho 3-18-15, Tokushima 770-8503, Japan. E-mail: kido@ier.tokushima-u.ac.jp.
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Abbreviations used
BU: Binding unit
CB: Cord blood
DLC: Diamond-like-carbon
MB: Maternal blood
NB: Neonatal blood

cleaning of 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, Franklin Lakes, NJ), and venous MB was obtained at 4 to 5 days after delivery. Blood samples were then centrifuged at 150 × *g* for 10 minutes to prepare serum. Serum was frozen at −30°C until analysis. All subjects provided written informed consent to participate in this study. This study was 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 IgG_4 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* and *Dermatophagoides pteronyssinus* were purchased from Allergon (Ängelholm, Sweden). Purified single allergens as molecular allergens, such as ovomucoid, ovalbumin, conalbumin, α-casein, β-casein, and β-lactoalbumin, were purchased from Sigma-Aldrich (St Louis, Mo). Japanese cedar was purchased from Cosmo Bio Co (Tokyo, Japan) and house dust from GREER (Lenoir, NC). Human serum IgE (75/502), IgG, IgA, and IgM (67/086) used as internal standards on chip were from the National Institute for Biological Standards and Control (Hertfordshire, United Kingdom).

After the activation of carboxylated DLC slides and the fabrication of allergen microarray, the individual arrays were incubated with 20 μL of 1:2 to 1:50 diluted serum as the primary antibody, then reacted with a HiLyte Fluor 555 (Dojindo Molecular Technologies, Inc, Kumamoto, Japan)-labeled secondary antibody against each human IgE, IgA, IgG, and IgG_4, and the resulting images were analyzed as described previously.[15] On each allergen chip, various concentrations of human IgE, IgG, or IgA were spotted as internal standards. From the cubic equation of IgE, IgG, or IgA standard concentrations, the amounts of allergen-specific antibodies bound to allergen on the chips were calculated and expressed as binding unit (BU). The BUs of IgE, IgA, IgG, and IgG_4 were reported as BUe, BUa, BUg, and BUg4, respectively. The detection limit of allergen-specific IgE against various natural and molecular allergens in serum in the DLC chip was 10 BUe/mL, which corresponds to about 0.07 IU/mL of the UniCAP system, indicating about 4 to 8 times higher sensitivity for the DLC chip than for the UniCAP system. The UniCAP system has a limit of 0.35 IU/mL for IgE detection.[6,16] The detection limits for allergen-specific IgA, IgG, and IgG_4 were 0.25 BUa, 2.50 BUg, and 0.53 BUg4, respectively.

We compared the sensitivity of the DLC chip with that of the UniCAP system for allergen-specific IgE in CB, which contains a relatively high level of nonspecific IgE antibodies[11,13,14] (Table I). The UniCAP system did not detect allergen-specific IgE antibodies in all CB samples analyzed in our experiments, even in samples of fluorescence units (BUe/mL) of more than 18 to 22 times the detection limit (10 BUe/mL) on the DLC chip. However, the difference in 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 by using an ELISA kit (Bethyl Laboratories, Montgomery, Tex) according to the protocol provided by the manufacturer. The chromogen produced was measured at an absorbance of 450 nm by using a SpectraMax Plus384 autoreader (Molecular Devices Corp, Sunnyvale, Calif).

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 the UniCAP system

	CB (1:1 dilution)		MB (1:1 dilution)	
Allergen	DLC chip (BUe/mL)	UniCAP (Ua/mL)	DLC chip (BUe/mL)	UniCAP (Ua/mL)
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 the measurement of allergen-specific IgE levels on the UniCAP system and the DLC chip. Detection limit on the DLC chip: 10 BUe/mL.
Df, Dermatophagoides farinae; *Dp, Dermatophagoides pteronyssinus*; *Ua*, arbitrary unit.
**ND*, Not detectable of UniCAP assay: <.35.

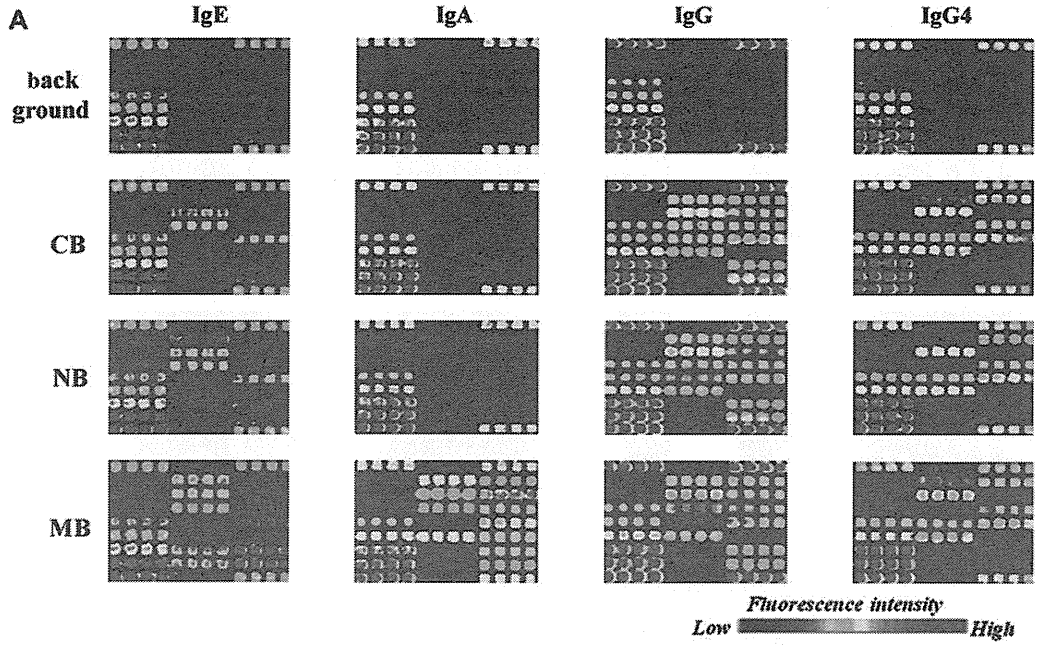
Statistical analysis

Statistical analysis was conducted by using the Statistical Package for Social Sciences (version18.0; SPSS, Inc, Chicago, Ill). 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 of ≤.05 was considered significant.

RESULTS

Allergen-specific serum IgE, IgA, IgG, and IgG_4 levels and their profiles in CB, NB, and MB

Allergen-specific IgE, IgA, IgG, and IgG_4 levels in CB, NB, and MB and their profiles were analyzed by using the allergen diagnosis DLC chips. The DLC chip detected more than 1 allergen-specific IgE of the tested 11 allergens in 83.7% of CB (n = 92). Fig 1 shows allergen-specific IgE, IgA, IgG, and IgG_4 profiles in representative paired CB, NB, and MB samples. MB contained high-reactive IgE levels against inhalant allergens *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae* and moderate-reactive IgE levels against food allergens milk, α-casein, and β-casein and also inhalant allergens cedar pollen and house dust. CB, however, did not contain any reactive IgE levels against inhalant allergens *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, cedar pollen, and house dust, but it had moderate-reactive IgE levels against food allergens milk, α-casein, β-casein, and ovomucoid. Although MB contained various allergen-specific IgA antibodies, CB did not show any reactive IgA. Almost identical levels of allergen-specific IgG and IgG_4 antibodies 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 antibodies in CB are derived from the fetus. The almost identical allergen-reactive



B

GD	Blank	GD
Blank	Milk	Egg white
Std	α-Casein	Egg yolk
	β-Casein	Ovalbumin
	α-Lactoalbumin	Ovomucoid
	β-Lactoglobulin	Conalbumin
	Cedar pollen	Dp
	House dust	Df
	Blank	GD

FIG 1. Multiallergen-specific profiling patterns of IgE, IgA, IgG, and IgG_4 in paired CB, NB, and MB analyzed by the DLC chip. **A,** Rainbow displays of fluorescence intensities of allergen-specific IgE, IgA, IgG, and IgG_4 reactivities against allergens of an array probed sera of paired CB, NB, and MB. **B,** Layout of the allergen DLC chip. *Df, Dermatophagoides farinae*; *Dp, Dermatophagoides pteronyssinus*; *GD,* guideline dot by standard serum; *Std,* standard calibration of human serum IgE from the National Institute for Biological Standards and Control. Each allergen was spotted in quadruple on the chip. Results are representative of 1 pair of 92 samples.

profiles of IgG and IgG_4 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.

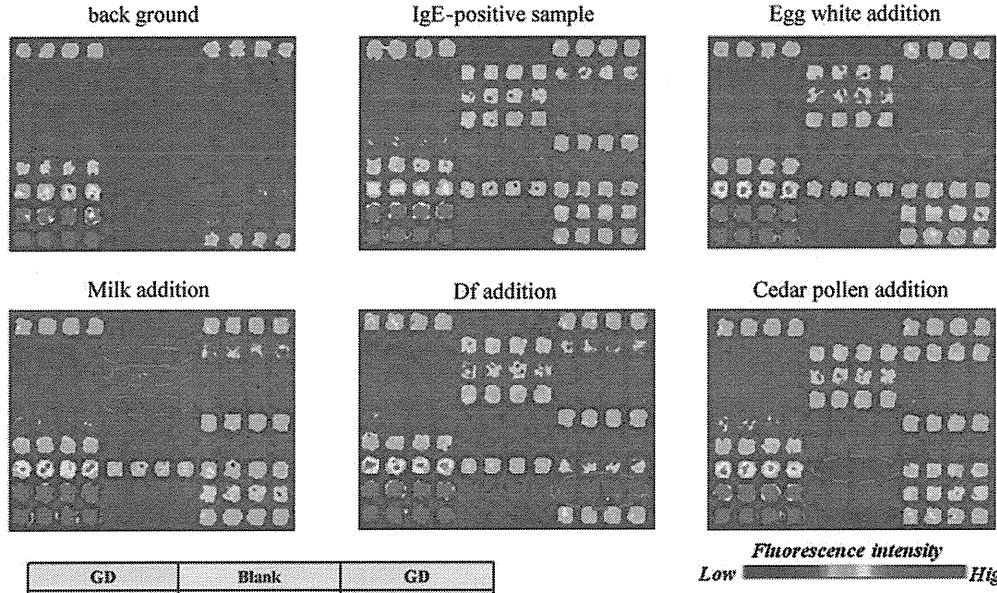
To evaluate the cross-reactivity of the antigen-IgE antibody reaction on the highly sensitive DLC chip, serum was preincubated with each allergen for 2 hours at 37°C followed by allergen-specific IgE detection on the chip (Fig 2). Each allergen selectively and almost completely adsorbed allergen-specific 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 the 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 using 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.

Allergen-specific IgE, IgA, IgG, and IgG_4 levels in CB and MB

Allergen-specific IgE, IgA, IgG, and IgG_4 levels were analyzed in 92 paired CB and MB samples. The proportion of CB samples positive for allergen-specific IgE against each food allergen (using a cutoff value of 10 BUe) ranged from 6.5% to 69.6%, with the highest proportion for ovomucoid, while the proportion of samples positive for each inhalant allergen ranged between 6.5% and 28.3% (Table II). The proportions of MB samples positive for allergen-specific IgE against each food allergen were almost similar to those of CB. In contrast, the proportions of MB samples positive for allergen-specific IgE against inhalant allergens were considerably higher (between 72.8% and 84.8%) than those of CB. Specifically, the proportion of CB samples positive for allergen-specific IgE



GD	Blank	GD
Blank	Milk	Egg white
Std	α-Casein	Egg yolk
	β-Casein	Ovalbumin
	α-Lactoalbumin	Ovomucoid
	β-Lactoglobulin	Conalbumin
	Cedar pollen	Dp
	House dust	Df
	Blank	GD

FIG 2. Allergen-specific IgE adsorption for evaluating cross-reactivity of antigen-IgE antibody reaction on the DLC chip. MB sample containing allergen-specific IgE against both food and inhalant allergens was preincubated with egg white (5 μg/mL), milk (5 μg/mL), Df (500 μg/mL), or cedar pollen (500 μg/mL) at 37°C for 2 hours. After the reaction, each sample was centrifuged at 17,500 × *g* for 30 minutes to remove antigen-antibody complex, and then the supernatant was used for multiallergen profiling of IgE on the DLC chip. Bottom, layout of the allergen on the DLC chip. *Df, Dermatophagoides farinae; Dp, Dermatophagoides pteronyssinus; GD,* guideline dot; *Std,* standard calibration of human serum IgE.

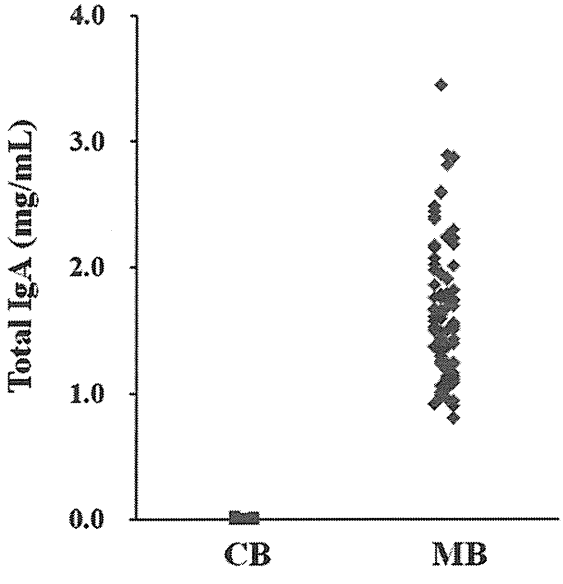


FIG 3. Total IgA levels in CB and MB. Total IgA levels in CB and MB (n = 92) were analyzed by ELISA.

with IgE-positive MB only against each food allergen was high and the mean value was 86.4%. The mean BU ratio for CB/MB in IgE-positive subjects only against the food allergens (ie, CB BUe/MB BUe; Table II) was 1.24 ± 0.60. In contrast, the proportion of CB samples positive for allergen-specific IgE in the IgE-positive MB subjects only against the inhalant allergens was lower (range, 9.0%-33.8%), with a mean value of 20.7%. The mean BU ratio for CB/MB in the IgE-positive subjects only against inhalant allergens (ie, CB BUe/MB BUe; Table II) was 0.54 ± 0.50. These results suggest a potentially greater sensitization against food allergens than against inhalant allergens *in utero*.

The levels of allergen-specific IgA against the allergens tested in CB were below the detection limits, but the proportion of MB samples positive for allergen-specific IgA against food and inhalant allergens ranged between 7.6% and 77.2%, with the highest value against milk (Table II). A fairly similar or identical trend was noted in CB and MB for allergen-specific IgG against food and inhalant allergens (Table III). The mean proportions of CB samples positive for allergen-specific IgG against food and inhalant allergens in subjects with IgG-positive MB only were 91.2% and 87.3%, respectively, and the mean BU ratios of CB/BM in the IgG-positive subjects only (CB BUg/MB BUg; Table III) against food and inhalant allergens were 1.08 ± 0.38 and 0.87 ± 0.24, respectively. The similar proportions of CB and MB samples and BU ratios around 1.0 indicate maternofetal transfer of allergen-specific IgG.

Although the mean proportion of allergen-specific IgG_4-positive CB in the IgG_4-positive MB subjects only against food

TABLE II. Allergen-specific IgE and IgA values in paired CB and MB samples, and BU ratio and correlation between CB- and MB-positive only

	CB (n = 92)		MB (n = 92)		CB/MB-positive only				
IgE positive in:	n	Percent*	n	Percent*	n	Percent†	BU ratio‡ (CB BUe/MB BUe ± SD)	Correlation between CB and MB (r_s)§	*P* value
Food									
α-Casein	28	30.4	25	27.2	18	72.0	1.09 ± 0.60	0.63	.005
β-Casein	16	17.4	15	16.3	11	73.3	1.10 ± 0.63	0.69	.002
α-Lactoalbumin	6	6.5	1	1.1	1	100	0.95 ± 0.00	NA	
β-Lactoglobulin	15	16.3	5	5.4	5	100	1.26 ± 0.65	0.30	.006
Ovalbumin	34	37.0	12	13.0	11	91.7	1.44 ± 0.70	0.44	.180
Ovomucoid	64	69.6	55	59.8	55	100	1.89 ± 0.82	0.89	<.001
Milk	18	19.6	22	23.9	12	54.5	1.14 ± 0.54	0.87	<.001
Egg white	23	25.0	8	8.7	8	100	1.06 ± 0.25	0.81	<.001
Mean						86.4	1.24 ± 0.60		
Inhalant									
Cedar pollen	6	6.5	67	72.8	6	9.0	0.34 ± 0.16	0.83	.042
Df	26	28.3	74	80.4	25	33.8	0.59 ± 0.58	0.06	.830
Dp	18	19.6	78	84.8	15	19.2	0.70 ± 0.74	0.21	.300
Mean						20.7	0.54 ± 0.50		

	CB (n = 92)		MB (n = 92)		CB/MB-positive only				
IgA positive in:	n	Percent*	n	Percent*	n	Percent†	BU ratio‡ (CB BUa/MB BUa ± SD)	Correlation between CB and MB (r_s)§	*P* value
Food									
α-Casein	0	NA	46	50.0	0	NA	NA	NA	
β-Casein	0	NA	50	54.3	0	NA	NA	NA	
α-Lactoalbumin	0	NA	7	7.6	0	NA	NA	NA	
β-Lactoglobulin	0	NA	15	16.3	0	NA	NA	NA	
Ovalbumin	0	NA	17	18.5	0	NA	NA	NA	
Ovomucoid	0	NA	19	20.7	0	NA	NA	NA	
Milk	0	NA	71	77.2	0	NA	NA	NA	
Egg white	0	NA	16	17.4	0	NA	NA	NA	
Mean									
Inhalant									
Cedar pollen	0	NA	21	22.8	0	NA	NA	NA	
Df	0	NA	35	38.0	0	NA	NA	NA	
Dp	0	NA	26	28.3	0	NA	NA	NA	
Mean									

Df, Dermatophagoides farinae; *Dp, Dermatophagoides pteronyssinus*; *NA,* not available due to lack of positive cases.
*Percentages of allergen-specific IgE- and IgA-positive samples.
†Percentages of allergen-specific IgE- and IgA-positive samples from MB-positive only.
‡BU ratios (CB BUe/MB BUe, CB BUa/MB BUa).
§Correlation coefficient analyzed Spearman's rank correlation test (r_s, *P* value) of allergen-specific IgE and IgA-positive CB and MB-positive only.

allergens was 96.0%, those against inhalant allergens was below the detection levels in the samples tested (Table III). The mean BU ratio between IgG_4-positive CB and IgG_4-positive MB subjects only (CB BUg4/MB BUg4) against food allergens was 0.99 ± 0.39. The results add support to the notion of maternofetal transfer of allergen-specific IgG_4.

Correlation of allergen-specific IgE, IgA, IgG, and IgG_4 between CB and MB

The results of correlation analysis of allergen-specific IgE, IgA, IgG, and IgG_4 levels (BU) between paired CB and MB samples (n = 92) determined by the DLC chip are shown in Table IV. There were strong correlations for allergen-specific IgE levels against food allergens between CB and MB with considerably high correlation coefficients (range, 0.53-0.94; $P < .001$), with the exception of weak correlation for α-lactoalbumin at 0.37 ($P < .001$). In contrast, the correlation coefficients for inhalant allergens were low (range, 0.01-0.30). In addition, there were strong correlations for allergen-specific IgE against crude allergens between CB and MB in the IgE-positive MB subjects only (Table II), such as milk, egg white, and cedar pollen, with high correlation coefficients of 0.87 ($P < .001$), 0.81 ($P < .001$), and 0.83 ($P < .042$), respectively. The correlation profiles of allergen-specific IgE against various food and inhalant allergens for CB and MB are depicted in Fig 4.

There were also significant and strong correlations for allergen-specific IgG against food and inhalant allergens between CB and MB ($r_s > 0.74$; $P < .001$), except allergen-specific IgG against cedar pollen and α-lactoalbumin (r_s, not available) and against β-lactoglobulin ($r_s = 0.58$; $P < .001$) (Table IV). There were also strong correlations for allergen-specific IgG_4 against food allergens between CB and MB ($r_s > 0.85$; $P < .001$), although that for allergen-specific IgG_4 against α-lactoalbumin was weaker ($r_s = 0.68$; $P < .001$). The correlation

TABLE III. Allergen-specific IgG and IgG_4 values in paired CB and MB samples, and BU ratio and correlation between CB- and MB-positive only

	CB (n = 92)		MB (n = 92)		CB/MB-positive only				
IgG positive in:	**n**	**Percent***	**n**	**Percent***	**n**	**Percent†**	**BU ratio‡ (CB BUg/MB BUg ± SD)**	**Correlation between CB and MB (r_s)§**	***P* value**
Food									
α-Casein	34	37.0	34	37.0	32	94.1	0.93 ± 0.30	0.75	<.001
β-Casein	7	7.6	8	8.7	7	87.5	0.88 ± 0.24	0.83	.021
α-Lactoalbumin	1	1.1	1	1.1	1	100	1.12 ± 0.00	NA	
β-Lactoglobulin	6	6.5	7	7.6	5	71.4	1.14 ± 0.44	0.90	.037
Ovalbumin	52	56.5	50	54.3	48	96.0	1.30 ± 0.60	0.80	<.001
Ovomucoid	47	51.1	46	50.0	45	97.8	1.18 ± 0.45	0.90	<.001
Milk	24	26.1	27	29.3	24	88.9	0.92 ± 0.26	0.53	.008
Egg white	52	56.5	52	56.5	49	94.2	1.15 ± 0.35	0.87	<.001
Mean						91.2	1.08 ± 0.38		
Inhalant									
Cedar pollen	0	0.0	1	1.1	0	NA	NA	NA	
Df	19	20.7	23	25.0	19	82.6	0.86 ± 0.20	0.71	.001
Dp	24	26.1	25	27.2	23	92.0	0.88 ± 0.28	0.83	<.001
Mean						87.3	0.87 ± 0.24		

	CB (n = 92)		MB (n = 92)		CB/MB-positive only				
IgG_4 positive in:	**n**	**Percent***	**n**	**Percent***	**n**	**Percent†**	**BU ratio‡ (CB BUg4/MB BUg4 ± SD)**	**Correlation between CB and MB (r_s)§**	***P* value**
Food									
α-Casein	34	37.0	33	35.9	33	100	0.93 ± 0.39	0.89	<.001
β-Casein	13	14.1	15	16.3	13	86.7	0.76 ± 0.27	0.95	<.001
α-Lactoalbumin	17	18.0	16	17.0	16	100	0.90 ± 0.42	0.92	<.001
β-Lactoglobulin	40	43.5	42	45.7	38	90.5	1.07 ± 0.42	0.93	<.001
Ovalbumin	68	73.9	67	72.8	67	100	1.19 ± 0.46	0.91	<.001
Ovomucoid	70	76.1	70	76.1	70	100	1.16 ± 0.48	0.93	<.001
Milk	24	26.1	25	27.2	23	92.0	0.76 ± 0.30	0.94	<.001
Egg white	66	71.7	67	72.8	66	98.5	1.15 ± 0.44	0.94	<.001
Mean						96.0	0.99 ± 0.39		
Inhalant									
Cedar pollen	0	NA	0	NA	0	NA	NA	NA	
Df	0	NA	0	NA	0	NA	NA	NA	
Dp	0	NA	0	NA	0	NA	NA	NA	
Mean									

Df, Dermatophagoides farinae; *Dp, Dermatophagoides pteronyssinus*; *NA*, not available due to lack of positive cases.

*Percentages of allergen-specific IgG- and IgG_4-positive samples.

†Percentages of allergen-specific IgG- and IgG_4-positive samples from MB-positive only.

‡BU ratios (CB BUe/MB BUe, CB BUa/MB BUa).

§Correlation coefficient analyzed Spearman's rank correlation test (r_s, *P* value) of allergen-specific IgE and IgA-positive CB and MB-positive only.

coefficients between CB and MB were not available for allergen-specific IgA against the allergens tested (Table II). Furthermore, the correlation coefficients for allergen-specific IgG_4 against inhalant allergens were not available because allergen-specific BUg4 values were below the detection levels (Tables III and IV). The correlation profiles for allergen-specific IgG and IgG_4 against each allergen between CB and MB are shown in Figs E1 and E2 in this article's Online Repository at www.jacionline.org.

DISCUSSION

We recently developed a new allergen diagnosis microarray with high sensitivity by using DLC-coated chips for profiling allergen-specific IgE, IgA, IgG, and IgG_4 against food and inhalant allergens. The DLC chip allows lowering the limit of detection of allergen-specific IgE in the UniCAP system to further dilution at 4- to 8-fold for each allergen,[15] and the detection limit of allergen-specific IgE in the DLC chip had about 5 times higher sensitivity than that in the UniCAP system in MB and serum of allergic patients. The present study demonstrated a larger difference in the detection sensitivity between the DLC chip and the UniCAP system in CB than in MB (Table I). The reason for the larger difference is not clear at this stage, but a relatively high level of nonspecific IgE in CB may disturb the detection of allergen-specific IgE on the UniCAP system but not on the DLC chip system. The latter immobilizes extremely high-density antigens on the surface of the DLC-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 the 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

TABLE IV. Correlation of allergen-specific IgE, IgG, and IgG_4 BU between the 92 paired CB and MB

	Correlation between CB and MB					
	IgE		IgG		IgG_4	
Allergen	r_s	*P* value	r_s	*P* value	r_s	*P* value
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	

Df, Dermatophagoides farinae; *Dp, Dermatophagoides pteronyssinus*; *NA*, not available due to lack of positive cases.

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. The measurement of the 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 immunoglobulins both in CB/NB and MB by using the newly developed highly sensitive allergen diagnosis DLC chip.

The allergen diagnosis DLC chip detected allergen-specific IgE against more than 1 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 that were not identical to those 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 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 antibodies 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 less than 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 IgG_4 (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 IgG_4 profiles in CB and MB provide support for 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 that of 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 transport.[29] Therefore, the presence of allergen-specific IgG levels in MB and CB may increase the risk of maternofetal allergen transfer and induction of allergen-specific IgE in CB.[30] Furthermore, it has been shown that the fetal immune system can produce IgE antibodies from week 11 of gestation,[13] and thus maternofetal transfer of allergen may trigger allergen-specific IgE production *in utero*. Once these food and inhalant allergens are transferred across the barrier, they may induce allergic sensitization *in utero* under the influence of maternal immune conditions.

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

Previous studies reported the presence of low (undetectable) levels of allergen-specific IgE in infant blood during the breast-feeding period at 6 months of age, compared with detectable levels of allergen-specific IgE in CB of some infants.[11,31] This observation might be due to the separation after birth from the source of allergens (ie, amniotic fluid and transplacental transport) and also from the maternal immune system. The findings of sequential appearance of first food-related and later in the preschool age of inhalant allergen-related IgE despite constant environmental exposure to the inhalant allergens by birth is a common knowledge. To study the mechanisms of age-dependent changes in the allergic phenotypes, simultaneous measurements of antigen-specific IgE, IgA, IgG_1, IgG_4, 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

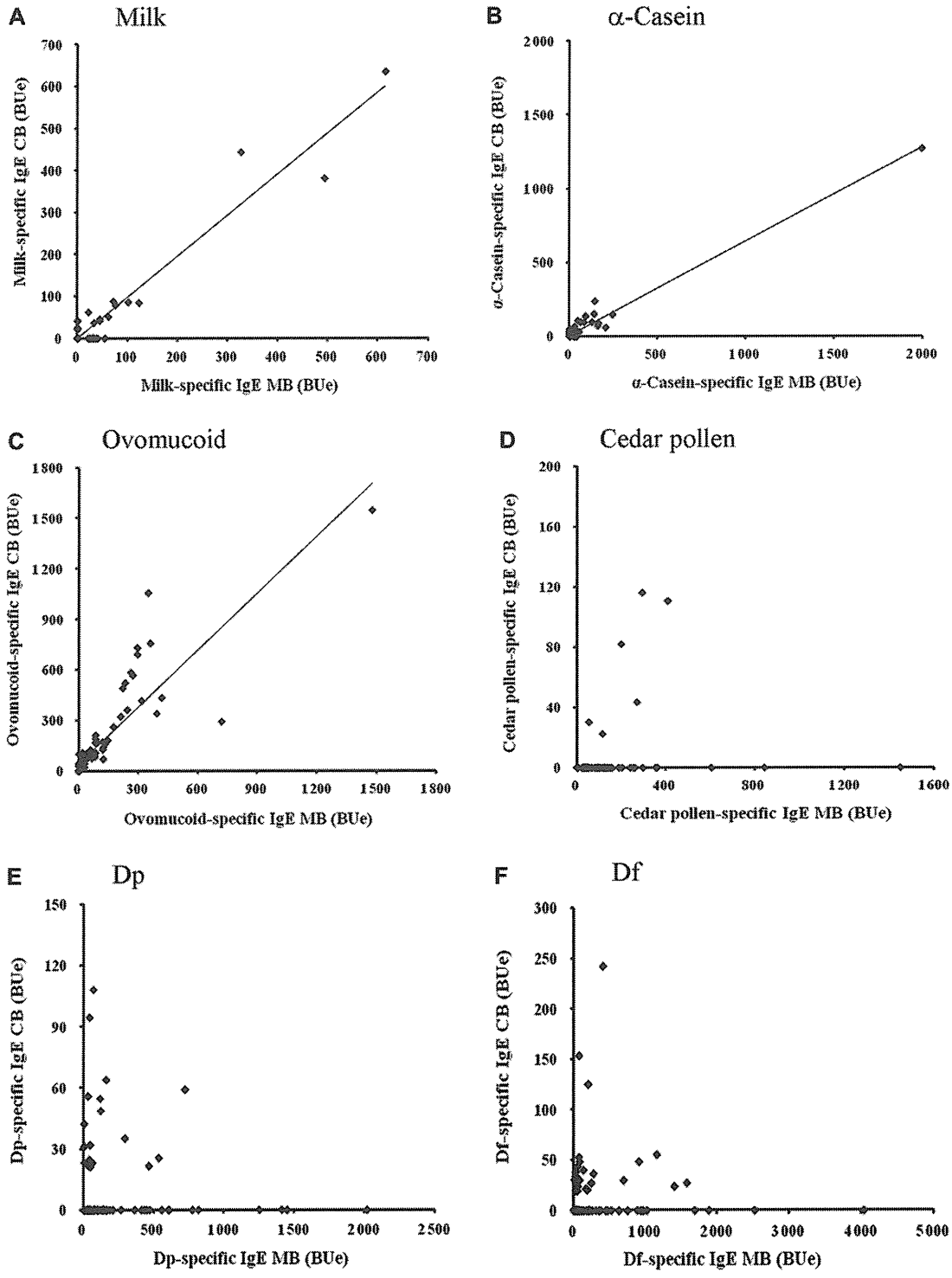


FIG 4. Comparison and correlation of allergen-specific IgE levels between CB and MB analyzed by the DLC chip. Allergen-specific IgE levels in CB and MB (n = 92) depicted in BUe. The cutoff value was 10 BUe. **A,** Milk. **B,** α-Casein. **C,** Ovomucoid. **D,** Cedar pollen. **E,** Dp. **F,** Df. Spearman's rank correlation test was used to assess the relation between the values of CB and MB. *Df, Dermatophagoides farinae; Dp, Dermatophagoides pteronyssinus.*

study found allergen-specific IgG against food and inhalant allergens but no allergen-specific IgG_4 against inhalant allergens in MB and CB. Further studies are also required on the relationship between allergen-specific IgE, IgG, and IgG_4 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.

We thank all mothers and their babies, as well as the nursing staff, obstetricians, and pediatricians at the Health Insurance Naruto Hospital, JFE Kenpo Kawatetsu Chiba Hospital, and Chiba University Hospital, who participated in this study. We also thank all field workers who were involved in data collection.

Clinical implications: There is evidence for intrauterine sensitization of allergen-specific IgE in cord blood analyzed by a highly sensitive new allergen diagnosis microarray.

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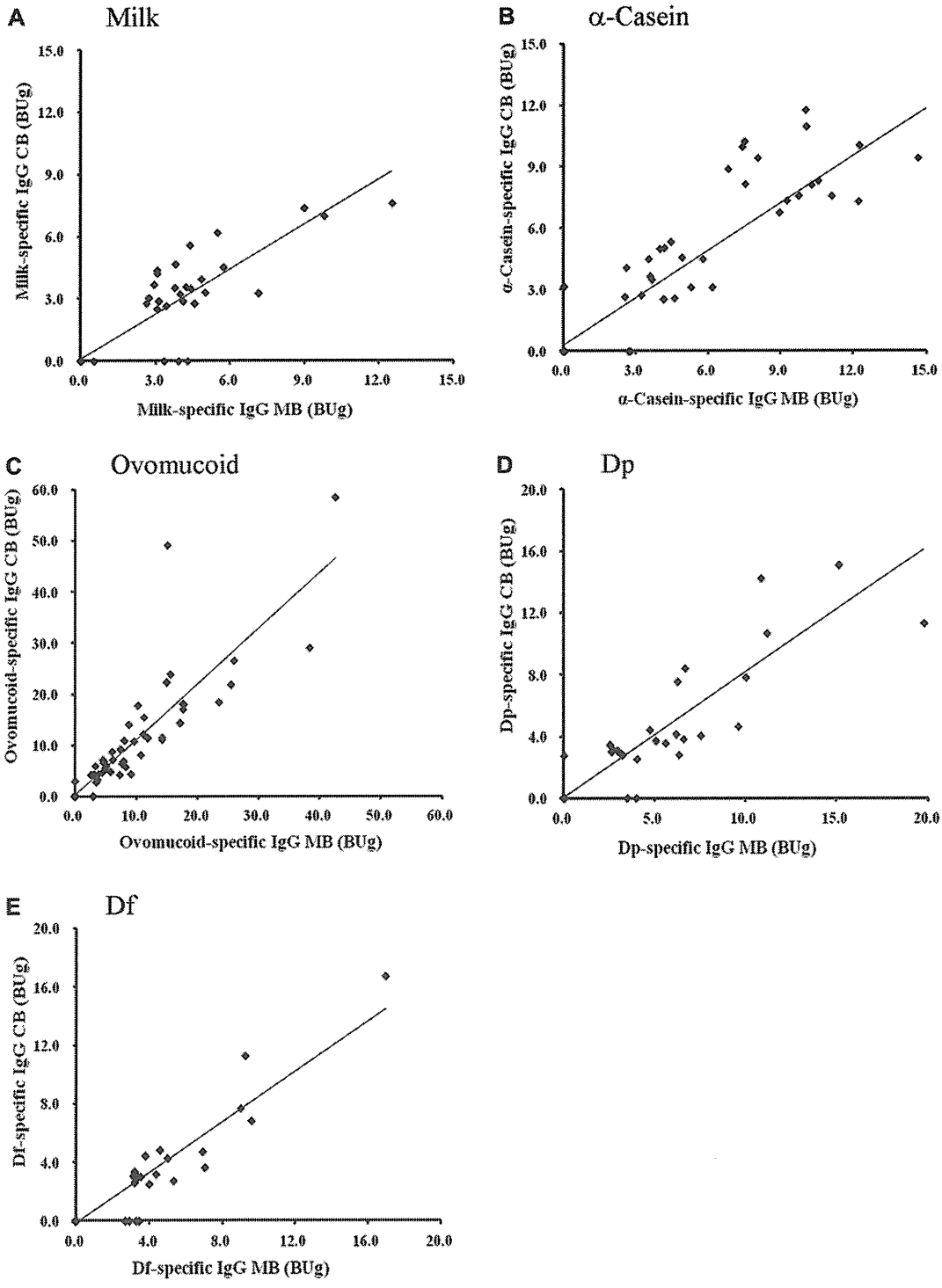

FIG E1. Comparison of allergen-specific IgG levels in CB and MB analyzed by the DLC chip. Allergen-specific IgG levels in paired samples of CB and MB (n = 92) depicted in BUg. The cutoff value was 2.50 BUg. **A,** Milk. **B,** α-Casein. **C,** Ovomucoid. **D,** Dp. **E,** Df. Spearman's rank correlation test was used to assess the relation between CB and MB. *Df, Dermatophagoides farinae; Dp, Dermatophagoides pteronyssinus.*

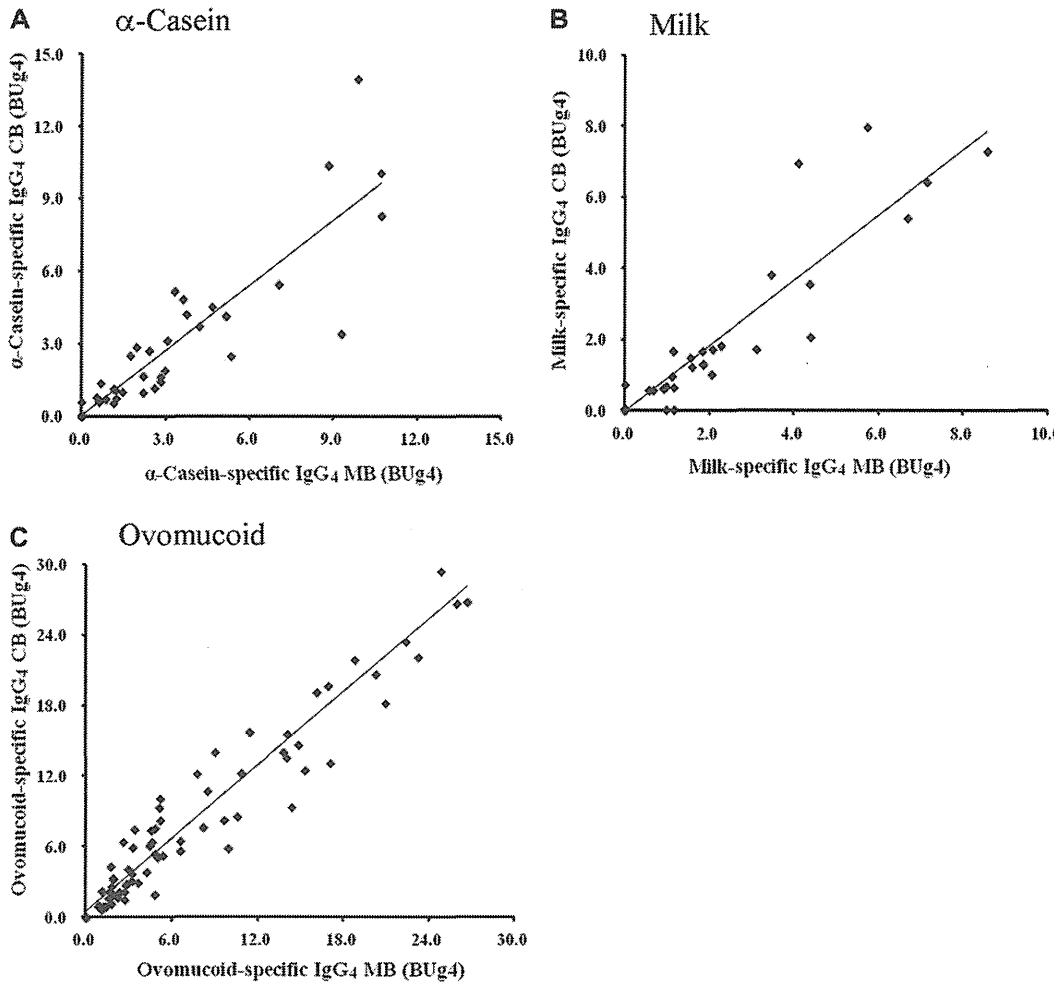

FIG E2. Comparison of allergen-specific IgG_4 levels in CB and MB analyzed by the DLC chip. Allergen-specific IgG_4 levels in paired samples of CB and MB (n = 92) depicted in BUg4. The cutoff value was 0.53 BUg4. **A,** α-Casein. **B,** Milk. **C,** Ovomucoid. Spearman's rank correlation test was used to assess the relation between CB and MB.

Research article

Severe dermatitis with loss of epidermal Langerhans cells in human and mouse zinc deficiency

Tatsuyoshi Kawamura,[1] Youichi Ogawa,[1] Yuumi Nakamura,[1] Satoshi Nakamizo,[2] Yoshihiro Ohta,[3] Hajime Nakano,[4] Kenji Kabashima,[2] Ichiro Katayama,[5] Schuichi Koizumi,[6] Tatsuhiko Kodama,[3] Atsuhito Nakao,[7] and Shinji Shimada[1]

[1]Department of Dermatology, Faculty of Medicine, University of Yamanashi, Yamanashi, Japan. [2]Department of Dermatology, Kyoto University Graduate School of Medicine, Kyoto, Japan. [3]Laboratory for Systems Biology and Medicine, Research Center for Advanced Science and Technology, University of Tokyo, Tokyo, Japan. [4]Department of Dermatology, Hirosaki University Graduate School of Medicine, Hirosaki, Japan. [5]Department of Dermatology, Osaka University School of Medicine, Osaka, Japan. [6]Department of Pharmacology and [7]Department of Immunology, Faculty of Medicine, University of Yamanashi, Yamanashi, Japan.

Zinc deficiency can be an inherited disorder, in which case it is known as acrodermatitis enteropathica (AE), or an acquired disorder caused by low dietary intake of zinc. Even though zinc deficiency diminishes cellular and humoral immunity, patients develop immunostimulating skin inflammation. Here, we have demonstrated that despite diminished allergic contact dermatitis in mice fed a zinc-deficient (ZD) diet, irritant contact dermatitis (ICD) in these mice was more severe and prolonged than that in controls. Further, histological examination of ICD lesions in ZD mice revealed subcorneal vacuolization and epidermal pallor, histological features of AE. Consistent with the fact that ATP release from chemically injured keratinocytes serves as a causative mediator of ICD, we found that the severe ICD response in ZD mice was attenuated by local injection of soluble nucleoside triphosphate diphosphohydrolase. In addition, skin tissue from ZD mice with ICD showed increased levels of ATP, as did cultured wild-type keratinocytes treated with chemical irritants and the zinc-chelating reagent TPEN. Interestingly, numbers of epidermal Langerhans cells (LCs), which play a protective role against ATP-mediated inflammatory signals, were decreased in ZD mice as well as samples from ZD patients. These findings suggest that upon exposure to irritants, aberrant ATP release from keratinocytes and impaired LC-dependent hydrolysis of nucleotides may be important in the pathogenesis of AE.

Introduction

Zinc (Zn) is a trace element essential for cell growth, development, and differentiation and is involved in maintaining the structure and function of over 300 different enzymes (1, 2). More than 2,000 transcription factors regulating gene expression require Zn for their structural integrity and binding to DNA (3). Recent studies revealed that Zn acts as an intracellular second messenger for transducing extracellular stimuli into intracellular signaling events in monocytes, DCs, and mast cells (4–7).

Zn deficiencies can be divided into 2 groups – a congenital form, called acrodermatitis enteropathica (AE; OMIM 201100), and the acquired forms (8). Recently, mutations in SLC39A4 have been identified as being responsible for congenital AE (9–11). SLC39A4 encodes ZIP4 Zn transporter, which is involved in Zn uptake via transporting Zn into the cytoplasm in intestine (9, 10). Congenital AE occurs worldwide, with an estimated incidence of 1 per 500,000 children, while it has been estimated that more than 2×10^9 people have a nutritional deficiency for Zn in developing countries (3, 8). It is even estimated that a considerable proportion of the Western population is at risk of marginal Zn deficiency (12, 13). Conditional Zn deficiencies also occur in many diseases and abnormal conditions, including malabsorption syndrome, chronic liver and renal diseases, sickle cell disease, excessive intake of alcohol, malignancies, and other chronic debilitating conditions (1, 3, 8).

The clinical manifestations of inherited and acquired Zn deficiency include growth retardation, diarrhea, alopecia, and characteristic skin lesions on acral, periorificial, and anogenital areas. Since Zn is indispensable for an adequate immunological response to all pathogens (14), the most serious complication observed in Zn deficiency is repeated infections due to impaired immune function. Indeed, several studies using animal models of Zn deficiency have confirmed that decreased levels of Zn induce thymic atrophy, lymphopenia, and compromised cell- and antibody-mediated immune responses (14, 15). Zn deficiency affects many aspects of immune function, including a shift of the Th cell response to a Th2 predominance, reduced antibody formation, reduced killing activity by NK cells and lower levels of phagocytosis and intracellular killing in granulocytes, monocytes, and macrophages (14–18). Zn also influences the production of chemokines and proinflammatory cytokines like TNF-α, IL-1β, and IL-6 (19–22).

The effects of Zn deficiency are particularly obvious in the skin and are seen as erythematous rashes, scaly plaques, and ulcers on acral and periorificial areas. Paradoxically, despite the impaired immune function in Zn deficiency, patients with hereditary and acquired AE present with immunostimulating skin inflammation, known as "acrodermatitis." It remains unclear which cellular processes induce this characteristic skin inflammation and account for the cutaneous pathological features of Zn deficiency (8). Here we investigated the mechanisms by which Zn deficiency induces dermatitis in AE using dietary Zn-deficient (ZD) mice.

Conflict of interest: The authors have declared that no conflict of interest exists.
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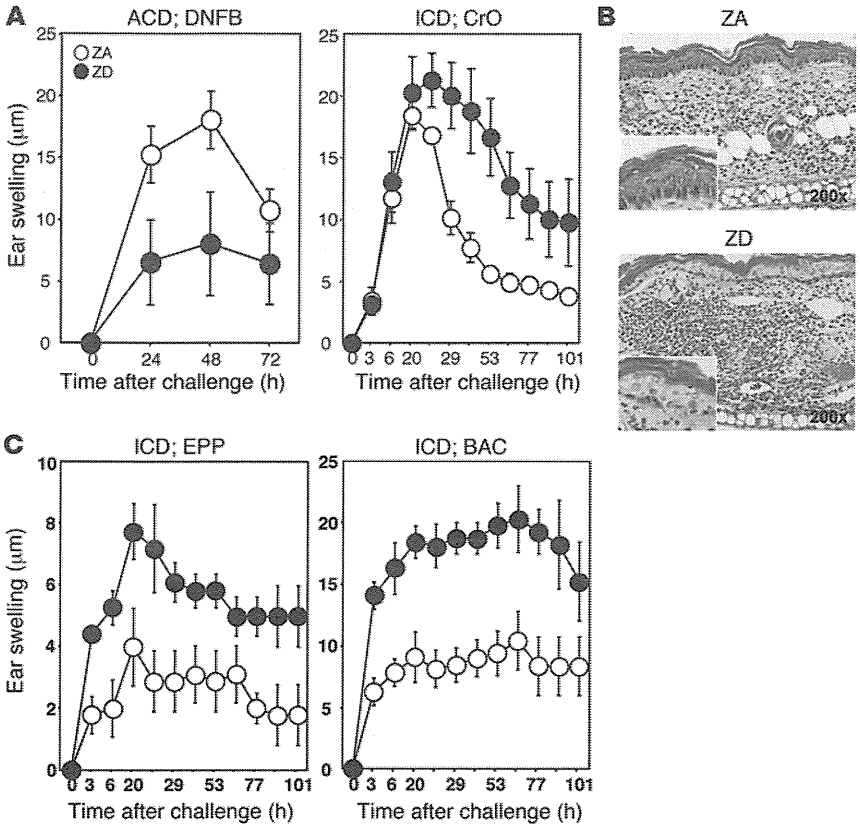

Figure 1
ZD mice demonstrate severe ICD with histological degeneration of keratinocytes. (**A** and **C**) ACD responses to DNFB and ICD responses to CrO, EPP, or BAC were induced in ZA (white circles) and ZD (black circles) mice, as described in Methods. Ear thickness was evaluated at the indicated time points. The data shown are (n = 5) the swelling responses (mean ± SD). (**B**) H&E staining of ear samples collected from ZA or ZD mice 24 hours after topical application of CrO. Original magnification: ×200; ×400 (insets). Data are representative of 3 independent experiments.

Results

Dietary Zn deficiency causes severe and prolonged irritant contact dermatitis with the histological features of AE. Symptoms of Zn deficiency in animals are similar across different species. Zn deficiency causes a well-characterized nutritional-immunological syndrome in mice (14–18, 23, 24), whereby young adults quickly manifest symptoms within 4 to 5 weeks of feeding a ZD diet containing 0.5 mg Zn/kg or more (23, 24). It is of note that, although ZD mice at approximately 70% to 80% of the control group weight exhibited visible cutaneous symptoms, such as alopecia and parakeratosis, they did not present with the characteristic inflammatory dermatitis of patients with AE (refs. 23, 24, and Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI58618DS1). Because AE in humans is typically seen on areas subject to repeated contact, we investigated allergic contact dermatitis (ACD) and irritant contact dermatitis (ICD) in dietary ZD mice. Five-week-old BALB/c mice were fed a ZD or Zn-adequate (ZA) diet, and at 10 weeks of age, ACD in response to dinitrofluorobenzene (DNFB) and ICD in response to croton oil (CrO) were quantified. Consistent with previous findings (24), ZD mice showed markedly decreased ear swelling responses to DNFB compared with those of ZA mice (Figure 1A). This was probably due to immunodeficiency in ZD mice, as previously reported (14, 15). Surprisingly, in contrast, the ear swelling response to CrO in ZD mice was significantly increased and prolonged compared with that of ZA mice (Figure 1A and Supplemental Figure 2). ICD caused by other skin irritant chemicals, benzalkonium chloride (BAC) and ethyl phenylpropiolate (EPP), was also tested, with similar results (Figure 1C and Supplemental Figure 2). Histological examination of ICD lesions in ZD mice revealed parakeratosis and cytoplasmic pallor, subcorneal vacuolization, and ballooning degeneration of keratinocytes and leukocyte infiltration (Figure 1B). These signs are histological features of cutaneous AE lesions in humans. No such degeneration of keratinocytes was observed in ICD lesions in ZA mice or ACD lesions in either ZA or ZD mice (Figure 1B and data not shown). These findings suggest that ICD, but not ACD, responses in ZD mice mimic the characteristic cutaneous manifestations observed in AE and can thus be considered an appropriate model for human disease.

Zn deficiency increases ATP release from keratinocytes in response to irritants. Accumulating evidence suggests that different environmental stimuli (e.g., chemical irritants) trigger adenosine 5′-triphosphate (ATP) release from keratinocytes via nonlytic mechanisms and also, more frequently, as a consequence of cell damage or acute cell death (25–27). Once released, ATP activates a family of plasma membrane receptors known as purinergic (P2) receptors. Because ATP released from chemically injured keratinocytes has been shown to cause ICD (28), we next compared the amount of ATP that was released from ZD or ZA mouse skin tissue after CrO application in ex vivo organ culture. Skin tissue obtained from ZD mice 4, 6, or 20 hours after CrO application released significantly greater amounts of ATP than tissues from ZA mice (Figure 2A). We next examined the effect of the Zn-chelating reagent TPEN on exogenous ATP release from Pam-212 keratinocytes cultured in vitro. Consistent with previous findings (26, 28), Pam-212 keratinocytes rapidly released ATP after exposure to CrO (Figure 2B). The addition of TPEN to the culture significantly augmented release of ATP in response to CrO, whereas TPEN alone failed to increase ATP release (Figure 2, B and D). The TPEN-mediated increase of ATP secretion was prevented by the addition of $ZnSO_4$ in a dose-dependent manner (Figure 2C). Furthermore, TPEN also significantly increased the secretion of ATP in response to other skin