ATOPIC DERMATITIS (AD), a chronic inflammatory skin disease that usually occurs in the first few years of life,[1] has increased dramatically in prevalence in developed countries over the past several decades. AD and other allergic diseases seem to have multifactorial origins, arising from complex interactions between genes and the environment; however, the relative importance of genetic and prenatal environmental factors is not yet clear. AD is one of the earliest manifestations of allergic/atopic diseases in children. Furthermore, AD is considered a significant risk factor for aeroallergen sensitization at 5 years and a predictor of the subsequent development of asthma.[2] Asthma is a worldwide problem, and the disease's social burden and costs to public and private health care systems are substantial. Therefore, establishing early risk factors for AD may help provide intervention strategies for the primary prevention of asthma.[3]

Cytokines are considered important regulators of functional maturation in the developing fetal immune system. However, factors that determine the degree of immune competence at birth and during early infancy are not fully understood. Several studies have linked cytokine profiles at birth to subsequent development of allergic disorders.[4–7] Tang et al[4] found that infants who either exhibited symptoms of atopic disease or had a positive skin test at 1 year of age produced significantly less interferon (IFN)-γ at birth than did infants without atopy. Macaubas et al[5] found negative relationships of interleukin (IL)-4, IFN-γ, and tumor necrosis factor (TNF)-α concentrations in cord blood to the risk of asthma, atopy, or both by 6 years of age. These findings suggested that some immune functions, including the capacity to secrete both T helper (Th) type 1 and 2 cytokines, are attenuated at birth in children who develop atopy subsequently. Most previous studies focused on the relationship between Th1/Th2 cytokines at birth and future development of atopic diseases. However, a few reports examined specifically links between levels of serial cytokines, which play roles in inflammation, maturation of T cells, and production and maintenance of the Th1/Th2 balance, at birth and future development of atopic diseases.

The pathogenesis of AD involves both allergic predisposition and nonallergic environmental factors. Skin barrier disruption has attracted attention as a nonallergic etiologic factor for AD, characterized by disorders of water retention and skin barrier function.[8] One study reported that levels of ceramide, a lipid contributing to skin function, were significantly lower in lesional and nonlesional skin of subjects with AD, compared with control subjects.[9] However, we know of no reported data concerning when in life dry skin and subsequent skin barrier disruption first become apparent in children who develop AD.

The purpose of the present prospective birth cohort study was to assess the risk of prenatal maternal factors and immunologic profiles at birth and skin functional parameters just after delivery and at 1 month of age in development of AD during the first year of life. To do this, we measured levels of 17 kinds of cytokines, including proinflammatory cytokines, Th1/Th2 distinguishing cytokines, nonspecifically acting cytokines, and chemokines, in serum from cord blood and measured stratum corneum hydration with an impedance meter until 5 days after delivery and again at 1 month.

METHODS

Subjects

This was a prospective cohort study examining multiple prenatal and perinatal factors in relation to child health outcomes. Participants were recruited at Ozawa Obstetric Clinic in Gunma Prefecture, where we explained the study to pregnant women and obtained informed consent at the routine clinic visit corresponding to 35 to 37 weeks of gestation. We enrolled 279 pregnant women (93.9% of eligible subjects) between June 1, 2002, and May 31, 2003. Only children born at term without significant neonatal respiratory difficulties or pathologic jaundice were included in the study, to avoid influences on infant skin physiologic features from humidification in an infant incubator or dehydration during phototherapy. These criteria excluded only 10 children.

Cord blood was collected from participants at birth. Blood was centrifuged at 3000 $\times$ *g* for 15 minutes, and serum was separated and stored at -30°C until cytokine measurement. Mothers were provided a self-administered questionnaire and interviewed briefly. Pregnancy and perinatal data were collected from perinatal records. The main factors taken into account were parental history of atopic disease (asthma, eczema, or hay fever), maternal age, viral infection (upper airway or gastrointestinal infection) or bacterial infection (urinary tract or vaginal infection attributable to *Escherichia coli*, *Chlamydia* spp, Gram-positive group B streptococcus, or other bacteria) during the prenatal period, gestational age, infant birth weight, and method of delivery. This study was approved by the committee of ethics at the Department of Pediatrics and Developmental Medicine, Gunma University Graduate School of Medicine.

Follow-up Examinations

In physical examinations at the 1-month checkup visit in our clinic, one of the authors noted the presence or absence of eczema on the face. At that time, physiologic skin measurements also were performed. Children were diagnosed as having infantile eczema when facial eczema was present at 1 month of age. All children were monitored for at least 1 year, for assessment of the development of atopic diseases such as AD, asthma-like illness, and food allergies. Parents were asked directly,

through mail or telephone interviews, whether they had been told by a physician that their child had AD. Information on lifestyle factors and other potential risk factors, including breastfeeding history (exclusive breastfeeding, partial breastfeeding, or milk formula feeding), also was collected with a parental questionnaire.

Skin Physiologic Measurements

Stratum corneum hydration was measured on the forehead, cheek, flexor aspect of the forearm near the cubital fossa, and chest at 5 to 10 hours after delivery and then once daily until 5 days after delivery. All tests were performed in open cribs in a controlled environment, with room temperature ranging from 22°C to 24°C and humidity at 50%. Skin temperature remained stable during the examination for all newborns. Stratum corneum hydration was measured by using a moisture meter (ASA-M1; Asahi Biomed, Tokyo, Japan),[10] based on capacitance and electrical conductance determined at 2 different frequencies (160 Hz and 143 kHz) with 2 concentric surface electrodes. The probe was pressed on the skin surface for 1 to 2 seconds. Each measurement was obtained twice at the same site; data were rejected when children were crying or visibly sweating. Parameters obtained were moisture content on the skin surface and moisture content in the stratum corneum.

Multiplex Cytokine Array Analysis

Multiplex cytokine array analysis was performed by using the Bio-Plex protein array system (Bio-Rad Laboratories, Hercules, CA), using Luminex-based technology.[11] With this assay, we quantitated cytokines simultaneously in serum from cord blood, including IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, and IL-17, IFN-γ, and TNF-α, granulocyte/macrophage colony-stimulating factor, granulocyte colony-stimulating factor, monocyte chemotactic protein (MCP)-1, and macrophage inflammatory protein (MIP)-1β. Most standard curves ranged between 0.2 pg/mL and 3200 pg/mL. At higher and lower concentrations, standard curves became flat and lost linearity. Lower limits of detection for the assays used were 0.2 pg/mL for all cytokines studied. Samples with undetectable concentrations were assigned a value of 0.1 pg/mL (ie, halfway between 0 and the lower limit of detection for the assay).

Statistical Methods

The relationship between eczema in infancy and various maternal and perinatal risk factors was assessed by using Pearson χ^2 tests. Differences in clinical characteristics at birth and in skin physiologic parameters between children with and without AD in the first year of life were analyzed by using Student's t tests. Because the distribution of cytokines is highly skewed, with many values below the lower limit of detection, the Mann-Whitney U test was used to compare cytokine concentrations in cord blood between children with and without AD or infantile eczema. Multivariate logistic regression models were used to determine independent effects of different factors associated with AD in this population; results are expressed as odds ratios (ORs) with 95% confidence intervals (CIs). Subject characteristics and prenatal factors that were statistically significant in Pearson χ^2 tests, Student's t tests, or Mann-Whitney U tests were included in the multivariate model.

RESULTS

Family History and Prenatal Factors

Children in this study were monitored prospectively, and questionnaire data from 213 (79%) were collected to assess the incidence of AD during the first year of life. The cumulative incidence of maternally reported, physician-diagnosed AD in the cohort was 27 cases (12.4%) among 213 children. Infant gender, method of delivery, length of gestation, birth weight, and maternal age did not differ between children with and without AD (Table 1). Exclusive breastfeeding was not related to the devel-

TABLE 1 Perinatal and Postnatal Characteristics of Infants With AD or Infantile Eczema

	AD			Infantile Eczema		
	Yes (N = 27)	No (N = 186)	P	Yes (N = 26)	No (N = 187)	P
Male/female, n	16/11	86/100	.206	17/9	85/102	.057
Cesarean section, n (%)	3 (11.1)	26 (14.0)	.916	5 (19.2)	121 (11.2)	.396
Gestational age, mean ± SD, wk	40.1 ± 1.2	39.9 ± 1.1	.343	39.8 ± 1.1	39.9 ± 1.1	.692
Birth weight, mean ± SD, g	3068 ± 310	3121 ± 337	.462	3119 ± 299	3114 ± 339	.935
Maternal age, mean ± SD, y	27.7 ± 3.0	28.2 ± 4.3	.534	27.7 ± 3.8	28.2 ± 4.2	.551
Breastfeeding, n (%)	14 (51.9)	102 (54.8)	.771	13 (50)	103 (55.1)	.626
Infantile eczema, n (%)	6 (22.2)	20 (10.8)	.089			
Maternal infection, n (%)						
Bacterial[a]	5 (18.5)	22 (11.8)	.135	2 (7.7)	22 (11.8)	.549
Viral[b]	11 (40.7)	81 (43.5)	.783	15 (57.7)	77 (41.2)	.111

[a] Urinary tract infection or vaginal infection with *Chlamydia* spp, *Escherichia coli*, group B streptococci, or other bacteria.

[b] Upper airway infection or viral gastroenteritis.

opment of AD (Table 1). Rates of maternal bacterial or viral infection during pregnancy did not differ between the 2 groups (Table 1). The diagnosis was >3 times as likely among infants born to mothers with a history of eczema, compared with those born to mothers with no such history. Paternal history of eczema did not influence the likelihood of AD, whereas paternal history of hay fever was associated with less occurrence of AD (Table 2).

The incidence of physician-diagnosed infantile eczema at the age of 1 month was 26 cases (12.1%) among 213 children (Tables 1 and 2). Infantile eczema was more common in male infants than in female infants, although this gender difference was not significant. Infants with infantile eczema were likely to develop AD during the first year (Table 1). Parental history of atopy was not related to the likelihood of infantile eczema (Table 2).

Cytokine Profiles

The proportions of samples with detectable cytokine concentrations were 22% for IL-2, 1% for IL-4, 87% for IL-6, 99% for IL-8, 46% for IL-10, 43% for granulocyte/macrophage colony-stimulating factor, 31% for IFN-γ, 55% for TNF-α, 52% for IL-1β, 36% for IL-5, 91% for IL-7, 16% for IL-12, 30% for IL-13, 48% for IL-17, 70% for granulocyte colony-stimulating factor, 100% for MCP-1, and 100% for MIP-1β. No additional analyses of IL-4 were performed, because of the small number of samples with detectable concentrations. Associations between cord blood cytokines and AD outcomes were analyzed first as a categorical variable (detectable or undetectable) and then in terms of concentrations. There was no difference in the categorical variable for each cytokine between children with and without AD (data not shown). Table 3 compares cytokine concentration profiles in cord blood samples from infants with or without development of AD during their first year. MIP-1β levels were significantly lower in samples from infants who developed AD than in those from infants who did not. A trend toward decreased IL-7 and MCP-1 concentrations in infants with AD fell short of significance (Table 3). Unlike AD, infants who developed infantile eczema had significantly higher cord blood concentrations of IL-5, IL-17, and MCP-1 than did those who did not (Table 3).

Skin Parameters

The moisture content of the skin surface and that in the stratum corneum were significantly lower on the forehead, cheek, chest, and forearm on postnatal days 2 to 4 than at the age of 1 month ($P < .0001$) (Table 4). The surface moisture content on the forehead, cheek, and forearm was significantly higher than that on the chest on postnatal days 2 to 4 and at the age of 1 month. The moisture content in the stratum corneum was significantly higher on the cheek than on the forehead, chest, or forearm on postnatal days 2 to 4 ($P < .0001$) and was significantly higher on the forearm than on the forehead, cheek, or chest at the age of 1 month ($P < .0001$).

No significant differences in the moisture content of the surface or in the stratum corneum were evident on postnatal days 2 to 4 between infants with and without subsequent AD. In contrast, the moisture content of the surface and stratum corneum of the forehead and cheek at 1 month of age was significantly greater for infants with AD than for those without AD. Infants who developed infantile eczema showed no difference from those who did not in surface or stratum corneum moisture content for any region or time point, except for greater moisture content on the surface of the cheek at the age of 1 month.

Multivariate logistic regression analysis was used to assess independent effects of the various postnatal and prenatal risk factors on development of AD (Table 5). All factors that approached significance with Pearson χ^2 tests, Student's t tests, or Mann-Whitney U tests were included in the model, that is, paternal hay fever, maternal eczema, MIP-1β levels, surface moisture content on the forehead and cheek, and moisture content of the stratum corneum of the forehead and cheek, all at the age of 1 month. Paternal hay fever (OR: 0.129; 95% CI: 0.020–0.845), MIP-1β levels (OR: 0.982; 95% CI: 0.967–0.998), and moisture content on the surface of

TABLE 2 Family History for AD and Infantile Eczema

	AD			Infantile Eczema		
	Yes (N = 27), n (%)	No (N = 186), n (%)	P	Yes (N = 26), n (%)	No (N = 187), n (%)	P
Paternal history						
Eczema	2 (7.4)	10 (5.4)	.669	2 (7.7)	10 (5.3)	.627
Asthma	1 (3.7)	8 (4.3)	.885	2 (7.7)	7 (3.7)	.348
Hay fever	2 (7.4)	61 (32.8)	.007	8 (30.8)	55 (29.4)	.887
Maternal history						
Eczema	4 (14.8)	8 (4.3)	.027	3 (11.5)	9 (4.8)	.163
Asthma	1 (3.7)	9 (4.8)	.794	1 (3.8)	9 (4.8)	.827
Hay fever	6 (22.2)	49 (26.3)	.647	6 (23.1)	49 (26.2)	.733

TABLE 3 Cytokines and Chemokines in Cord Blood According to Development of AD During the First Year and Infantile Eczema During the First Month

	Concentration, Median (Interquartile Range), pg/mL					
	AD			Infantile Eczema		
	Yes	No	*P*	Yes	No	*P*
IL-2	0.10 (0.10–0.65)	0.10 (0.10–0.10)	.7005	0.10 (0.10–1.50)	0.10 (0.10–0.10)	.6092
IL-6	4.19 (2.18–10.45)	5.67 (1.80–13.34)	.6149	7.86 (3.52–16.40)	5.35 (1.64–12.42)	.1106
IL-8	32.4 (3.3–312.2)	34.5 (4.0–253.1)	.8321	69.5 (9.6–1020.7)	31.6 (3.2–246.5)	.1316
IL-10	0.10 (0.10–0.19)	0.10 (0.10–0.53)	.1939	0.10 (0.10–0.49)	0.10 (0.10–0.48)	.834
GM-CSF	0.10 (0.10–4.02)	0.10 (0.10–6.60)	.2826	2.47 (0.10–19.01)	0.10 (0.10–4.88)	.1719
IFN-γ	0.10 (0.10–0.78)	0.10 (0.10–0.83)	.8562	0.10 (0.10–0.77)	0.10 (0.10–0.83)	.9786
TNF-α	0.18 (0.10–0.65)	0.18 (0.10–0.74)	.7769	0.33 (0.10–0.79)	0.18 (0.10–0.66)	.4371
IL-1β	0.39 (0.10–1.78)	0.23 (0.10–2.07)	.8969	0.91 (0.10–2.34)	0.15 (0.10–2.00)	.3643
IL-5	0.10 (0.10–0.13)	0.10 (0.10–0.21)	.2485	0.13 (0.1–0.43)	0.10 (0.10–0.17)	.0277
IL-7	0.83 (0.51–1.19)	1.02 (0.64–1.97)	.0783	1.33 (0.68–2.72)	0.96 (0.59–1.65)	.1675
IL-12	0.10 (0.10–0.10)	0.10 (0.10–0.10)	.6068	0.10 (0.10–0.10)	0.10 (0.10–0.10)	.9702
IL-13	0.10 (0.10–0.13)	0.10 (0.10–0.17)	.9104	0.10 (0.10–0.20)	0.10 (0.10–0.17)	.8855
IL-17	0.10 (0.10–1.25)	0.10 (0.10–1.95)	.1791	0.99 (0.10–2.87)	0.10 (0.10–1.36)	.0347
G-CSF	2.53 (8.72–5.86)	2.60 (0.10–8.16)	.7584	4.06 (0.81–13.29)	2.52 (0.10–6.78)	.3418
MCP-1	12.6 (9.0–22.8)	19.4 (10.4–72.9)	.0628	54.8 (17.0–85.6)	16.1 (9.8–63.1)	.0362
MIP-1β	57.3 (32.0–78.7)	64.0 (42.2–161.6)	.0233	110 (43–287)	62 (39–123)	.1422

GM-CSF indicates granulocyte/macrophage colony-stimulating factor; G-CSF, granulocyte colony-stimulating factor.

TABLE 4 Skin Parameters at 2 Days After Delivery and at 1 Month, According to Development of AD During the First Year and Infantile Eczema During the First Month

	AD			Infantile Eczema		
	Yes	No	*P*	Yes	No	*P*
Surface moisture, mean ± SD, μS						
Forehead						
2 d	0.47 ± 0.49	0.43 ± 0.50	.7165	0.43 ± 0.40	0.44 ± 0.51	.9196
1 mo	1.88 ± 0.97	1.44 ± 0.53	.0016	1.68 ± 0.78	1.46 ± 0.57	.0931
Cheek						
2 d	0.58 ± 0.49	0.49 ± 0.28	.1728	0.39 ± 0.17	0.51 ± 0.32	.0697
1 mo	1.72 ± 0.86	1.24 ± 0.43	<.0001	1.63 ± 0.78	1.24 ± 0.45	.0004
Chest						
2 d	0.30 ± 0.21	0.32 ± 0.25	.7064	0.30 ± 0.17	0.32 ± 0.25	.6789
1 mo	1.07 ± 0.31	1.02 ± 0.30	.4604	1.03 ± 0.27	1.03 ± 0.30	.9609
Forearm						
2 d	0.57 ± 0.56	0.61 ± 0.62	.7641	0.69 ± 0.64	0.59 ± 0.60	.4786
1 mo	1.54 ± 0.60	1.40 ± 0.50	.2532	1.41 ± 0.44	1.42 ± 0.44	.9674
Stratum corneum moisture, mean ± SD, μS						
Forehead						
2 d	13.5 ± 3.6	14.2 ± 5.8	.5435	14.2 ± 3.8	14.1 ± 5.8	.9681
1 mo	36.2 ± 9.0	32.5 ± 7.8	.0461	34.2 ± 9.5	32.7 ± 7.8	.3939
Cheek						
2 d	18.3 ± 4.9	17.0 ± 6.1	.3068	16.3 ± 4.3	17.3 ± 6.2	.457
1 mo	35.6 ± 6.5	31.3 ± 7.1	.0080	32.6 ± 5.8	31.7 ± 7.3	.5526
Chest						
2 d	13.9 ± 2.7	13.8 ± 3.1	.8630	14.2 ± 3.0	13.8 ± 3.0	.5289
1 mo	29.9 ± 7.2	28.3 ± 5.7	.2208	27.8 ± 5.9	28.6 ± 5.9	.5467
Forearm						
2 d	13.8 ± 5.9	14.7 ± 3.4	.5385	16.4 ± 7.5	14.3 ± 6.1	.1188
1 mo	45.2 ± 11.4	41.3 ± 9.4	.0852	43.4 ± 9.9	41.5 ± 9.6	.3705

the cheek at an age of 1 month (OR: 3.189; 95% CI: 1.279–7.952), but no other factors, were significant predictors of AD in the best-fitting model.

DISCUSSION

Development of atopic diseases is thought to depend on a complex interplay of genetic factors, environmental

TABLE 5 Multivariate Logistic Regression Results Concerning AD During the First Year of Life

	OR (95% CI)	*P*
Paternal hay fever	0.129 (0.020–0.845)	.0327
Maternal eczema	2.363 (0.45–12.55)	.3127
MIP-1β level	0.982 (0.967–0.998)	.0261
Surface moisture		
Forehead, 1 mo	2.118 (0.917–4.892)	.0787
Cheek, 1 mo	3.189 (1.279–7.952)	.0128
Stratum corneum moisture		
Forehead, 1 mo	0.99 (0.914–1.073)	.8107
Cheek, 1 mo	1.039 (0.958–1.126)	.3534

exposure to allergens, and nonspecific adjuvant factors. Only a few prospective birth cohort studies have addressed AD occurring in the first year of life.[6,7] We found several perinatal predictors of increased risk, including certain physiologic skin parameters, low cytokine concentrations in cord blood, and family history of atopic diseases.

We determined the cumulative incidence of maternally reported, physician-diagnosed AD in the cohort of infants studied to be 27 cases (12.4%) among 213 children, in essential agreement with other epidemiologic studies in the United Kingdom[12] and the German Multicenter Atopy Study,[13] which showed that 14% and 13.4% of subjects, respectively, developed AD in the first year of life. Our study demonstrated increased risk of AD for infants born to mothers with a history of eczema, which is consistent with previous reports that a positive maternal history predicted greater risk for childhood eczema than did a positive paternal history.[14] Atopy may be inherited preferentially through the maternal line or mothers may carry relatively more of the predisposing genes. Interestingly, we found a negative relationship between AD and paternal history of hay fever caused by Japanese cedar pollen. Genetic susceptibility to hay fever apparently does not contribute to the development of AD in children; indeed, paternal hay fever might provide a child with some protection against developing AD. Kurzius-Spencer et al[7] similarly found evidence of an apparent protective effect of paternal asthma, but mechanisms underlying these protective effects remain unknown.

Environmental influences during pregnancy and early life, particularly those related to hygiene and infections, seem to increase risks of asthma and allergic disease. In fact, certain infectious complications during pregnancy, such as respiratory tract infections, have shown associations with the development of asthma in childhood.[15,16] However, we could not detect associations between maternal viral or bacterial infections and the development of AD in children. This disagreement might involve differences in prenatal and postnatal actions of risk factors between various allergic diseases. For example, Calvani et al[17] demonstrated that most such factors affected mainly the risk of nonatopic but not atopic asthma. Another possible explanation is that our study was prospective, whereas most others had a cross-sectional design.

In the present study, cord blood concentrations of IL-6, IL-8, IL-10, granulocyte/macrophage colony-stimulating factor, TNF-α, IL-1β, IL-7, IL-17, granulocyte colony-stimulating factor, MCP-1, and MIP-1β were measurable in >40% of samples. However, other cytokines rarely were present in detectable amounts. The reason for the small number of samples with detectable concentrations of these cytokines might be related to suboptimal methods used for their quantification, because assays for some of the cytokines are known to be technically difficult. Alternatively, it is possible that various immune functions, including the capacity to secrete some cytokines, are attenuated at birth.[5,18] We could perform no additional analyses of IL-4 because the proportion of samples with detectable concentrations was only 1%.

In the present study, we found negative relationships between cord blood concentrations of MIP-1β and the risk of AD during the first year of life. We know of no previous reports suggesting that MIP-1β within the fetoplacental unit might influence susceptibility to subsequent disease development. The ability of chemokines to regulate Th1 and Th2 responses suggests that these mediators may take part in the pathogenesis of atopic diseases such as allergic asthma, for which Th2 response dominance has been observed. Influences of MIP-1α, MIP-1β, and regulated on activation, normal T cell expressed and secreted in the establishment of Th1 responses have been reported.[19] Grob et al[20] demonstrated intracellular expression of MIP-1β in $CD4^+$ and $CD8^+$ T cells from patients with allergic asthma to be significantly less than that in cells from subjects without asthma. This observation of diminished MIP-1β production by both $CD4^+$ and $CD8^+$ T cells suggests the relevance of this chemokine to disease development, with relative deficiency being likely to reflect dominance of Th2 responses over Th1 responses at the chemokine level. This view was supported by our present findings showing that low concentrations of cord blood MIP-1β were related to the risk of AD during the first year of life.

Infants with AD showed a trend toward lower concentrations of IL-7 and MCP-1, although the trend did not reach significance. Schonland et al[21] demonstrated IL-7 to be a powerful stimulator of neonatal T cells, driving most $CD4^+$ and $CD8^+$ T cells into the cell cycle. Furthermore, the combinations of IL-4 and IL-12 with IL-7 were found to provide superior enhancement of antigen-specific T cell proliferation.[22] Although MCP-1 was originally described for its chemotactic activity on monocytes, in vitro studies revealed an even higher activity on T cells.[23] Low concentrations of IL-7 and MCP-1 at birth may lead to impairment of T cell activa-

tion; therefore, infants may develop AD later during the first year of life. Another possible explanation for the lower cytokine levels might be secondary phenomena attributable to a different pattern of specific cell subtypes within the blood of these children. Additional studies are needed to confirm whether infants with AD have significantly lower concentrations of IL-7 and MCP-1 at birth.

In 1997, the European Group for Efficacy Measurements on Cosmetics and Other Topical Products gave recommendations regarding electrical measurement methods.[24] According to those recommendations, both single-frequency and multifrequency instruments may be used to assess skin hydration. High-frequency measurements in general reflect the deeper living layers of the skin, whereas low-frequency measurements are dominated by the stratum corneum. In the present study, we used a novel moisture meter (ASA-M1) that measures electrical admittance and susceptance at different excitatory frequencies.[10] We found significant differences between functional skin variables in neonatal life and infancy at the age of 1 month, as well as differences between regions for both neonates and infants. This suggests that stratum corneum function was still adapting to extrauterine life during the period studied. A similar conclusion was drawn when skin surface capacitance and electrical conductance were examined in newborns.[25] Hoeger and Enzmann[26] found a significant increase in stratum corneum hydration, paralleled by a decrease in skin roughness, in serial measurements at 3 days, 4 weeks, and 12 weeks of age.

To our knowledge, no studies have monitored skin function parameters prospectively, to compare directly children with and without subsequent development of AD. In the present study, we found no differences in the moisture content of the surface or stratum corneum, measured a few days after delivery, between infants with and without development of atopy. In contrast, we found significant differences in the moisture content of the surface and stratum corneum on the face at the age of 1 month. Only 6 of 27 infants who developed AD during their first year had facial infantile eczema at 1 month. Although the other 21 infants had no eczema on the face, certain differences in skin function parameters were demonstrated at 1 month between infants with and without subsequent AD. These data suggest that differences in skin physiologic features between infants with and without AD emerge during the first month of life but not in the first few days. We do not know the mechanisms underlying changes in skin function parameters in infants with AD during the first month of life, but abnormalities during this period may be related to impairment of skin adaptation to extrauterine life.

Dry skin, leading to skin barrier disruption, has attracted attention as a nonallergic etiologic factor in AD.[9] Several studies have demonstrated lower water-holding capacity in visually "uninvolved" skin of children with AD, compared with children without AD.[8] Contrary to our expectation, infants with AD had more moisture content in the surface and stratum corneum at the age of 1 month than did infants without AD. The earliest lesions of infantile AD are erythematous weepy patches on the cheeks, with subsequent extension to the rest of the face and neck. With increasing age, there is a tendency toward drying and thickening of the skin in the involved areas. Therefore, our findings at the age of 1 month may be consistent with the earliest lesions in the clinical course of infantile AD. Furthermore, Yosipovitch et al[25] found a positive relationship between stratum corneum hydration, as evaluated with capacitance measurements, and transepidermal water loss (which reflects skin barrier function in newborns), which indicates that electrical properties of newborn skin may provide an indirect measurement of transepidermal water loss; this was also suggested by Saijo and Tagami[27] and Okah et al.[28] It will be necessary to monitor the skin physiologic features of these infants during the first year, to understand when the findings may eventually change into dry skin, leading to the defective skin barrier that is known in AD.

Infants with infantile eczema at the age of 1 month showed differences in immunologic and skin physiologic parameters, compared with findings for infants with later development of AD during the first year of life. In fact, infants with infantile eczema showed significantly higher concentrations of IL-5, IL-17, and MCP-1. These data suggest that the intrauterine environment is more likely to reflect the development of infantile eczema, rather than that of AD. Recent data indicate that the proinflammatory cytokine IL-17 stimulates the recruitment and activation of neutrophils and macrophages.[29] Furthermore, IL-17 regulates expression of adhesion molecules and chemokines in keratinocytes, which participate actively in skin inflammatory diseases.[30] MCP-1 has been shown to induce the migration of monocytes, which form a significant component of the inflammatory reaction taking place in the skin. Accordingly, higher concentrations of IL-17 and MCP-1 in cord blood of infants who later develop infantile eczema at the age of 1 month may contribute to the enhancement of inflammatory reactions in the skin of these infants. Infants who developed infantile eczema showed no difference from those who did not in stratum corneum moisture content for any region or time point, whereas a significant difference was seen for infants who developed AD during their first year. The reason for the difference in skin physiologic parameters for infants with eczema at the age of 1 month and infants with later development of AD during the first year of life remains unknown. However, measurements of skin physiologic parameters seem to be useful to distinguish infants with later devel-

opment of AD from those with infantile eczema at the age of 1 month.

CONCLUSIONS

Our findings indicated that development of AD in infancy may be related to a decrease in MIP-1β production at birth, greater skin moisture in the surface of the cheek at 1 month of age, and paternal hay fever, in multivariate logistic regression analysis. The majority of risk factors had different effects on infant eczema and AD, which indicates different causes. The number of samples investigated in the present study might be small; larger prospective studies would serve to confirm our results and to explain the possible mechanism of how these factors act.

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Th1 and Type 1 Cytotoxic T Cells Dominate Responses in T-bet Overexpression Transgenic Mice That Develop Contact Dermatitis[1]

Kazusa Ishizaki,[2]* Akiko Yamada,[2]* Keigyou Yoh,[‡] Takako Nakano,* Homare Shimohata,* Atsuko Maeda,* Yuki Fujioka,* Naoki Morito,* Yasuhiro Kawachi,[§] Kazuko Shibuya,[†] Fujio Otsuka,[§] Akira Shibuya,[†] and Satoru Takahashi[3]*

Contact dermatitis in humans and contact hypersensitivity (CHS) in animal models are delayed-type hypersensitivity reactions mediated by hapten-specific T cells. Recently, it has become clear that both CD4$^+$ Th1 and CD8$^+$ type 1 cytotoxic T (Tc1) cells can act as effectors in CHS reactions. T-bet has been demonstrated to play an important role in Th1 and Tc1 cell differentiation, but little is known about its contribution to CHS. In the present study, we used C57BL/6 mice transgenic (Tg) for T-bet to address this issue. These Tg mice, which overexpressed T-bet in their T lymphocytes, developed dermatitis characterized by swollen, flaky, and scaly skin in regions without body hair. Skin histology showed epidermal hyperkeratosis, neutrophil, and lymphocyte infiltration similar to that seen in contact dermatitis. T-bet overexpression in Tg mice led to elevated Th1 Ig (IgG2a) and decreased Th2 Ig (IgG1) production. Intracellular cytokine analyses demonstrated that IFN-γ was increased in both Th1 and Tc1 cells. Furthermore, Tg mice had hypersensitive responses to 2,4-dinitrofluorobenzene, which is used for CHS induction. These results suggest that the level of expression of T-bet might play an important role in the development of contact dermatitis and that these Tg mice should be a useful model for contact dermatitis. *The Journal of Immunology,* **2007, 178: 605–612.**

The Th1/Th2 paradigm proposed by Mosmann et al. (1) holds that CD4$^+$ T cells can be subdivided into two categories, namely Th1 and Th2 (2). These two polarized subsets can be identified on the basis of the cytokines they secrete (3). Th1 cells produce IL-2 and IFN-γ, whereas Th2 cells produce IL-4, IL-5, IL-6, IL-10, and IL-13. More recently, a similar heterogeneity among CD8$^+$ T cytotoxic (Tc)[4] cells has also been recognized with the identification of Tc1 and Tc2 subpopulations (4, 5). IFN-γ is also one of the main cytokines produced by differentiated CD8$^+$ effector T cells and has been shown to have a fundamental role in CD8$^+$ T cell-mediated immunity (6). Lineage commitment of CD4$^+$ and CD8$^+$ T cells is transcriptionally regulated, often by the same factors that mediate T cell effector function.

T-bet is known as a Th1 lineage commitment transcription factor as a result of its transactivation of the Th1 cytokine IFN-γ (7). Recently, T-bet has also been shown to regulate cytolytic effector mechanisms of CD8$^+$ T cells (8). T-bet expression is rapidly induced in CD8$^+$ T cells by signaling through the TCR and the IFN-γR, and it functions downstream of STAT1 (6, 9, 10). In the context of Ag-specific activation, T-bet is required for the differentiation of naive CD8$^+$ T cells into effector CTLs.

Contact dermatitis is one of the most common skin diseases (11). Knowledge of the pathophysiology of contact dermatitis is derived chiefly from animal models in which the inflammation induced by hapten painting of the skin is referred to as contact hypersensitivity (CHS) (12). Contact dermatitis and CHS are delayed-type hypersensitivity reactions that are mediated by hapten-specific T cells (12). Skin sensitization resulting in contact dermatitis and CHS is dependent on the initiation of specific T lymphocyte responses (11, 13). Until recently it was believed that the most important cells in these responses were CD4$^+$ T lymphocytes. IL-2 and IFN-γ produced by Th1 cells are thought to play a preeminent role in the evolution of CHS (14, 15). Some investigations in mice found CHS to be associated with CD4$^+$ T lymphocyte function and to be compromised when such cells were deleted (15, 16). However, there is growing evidence that in many instances the predominant effector cell in CHS may be a CD8$^+$ T lymphocyte (13, 17, 18). Wang et al. (17) clearly demonstrated that the deletion of CD8$^+$ Tc1 cells had a more significant suppressive effect than the deletion of CD4$^+$ Th1 cells in CHS responses to 2,4-dinitrofluorobenzene (DNFB). According to these results, both CD4$^+$ Th1 and CD8$^+$ Tc1 cells are key players in CHS.

Although T-bet plays an important role in Th1 and Tc1 cell induction, little is known about its contribution to CHS. In the present study we used T-bet overexpression in T cell transgenic (Tg) mice to address this issue.

*Department of Anatomy and Embryology, [†]Department of Immunobiology, Biomolecular, and Integrated Medical Sciences, [‡]Department of Pathophysiology of Renal Diseases, and [§]Department of Dermatology, Medical Sciences for Control of Pathological Processes, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Ibaraki, Japan

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[2] K.I. and A.Y. contributed equally to this work.

[3] Address correspondence and reprint requests to Dr. Satoru Takahashi, Department of Anatomy and Embryology, Biomolecular, and Integrated Medical Sciences, Graduate School of Comprehensive Human Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki, 305-8575, Japan. E-mail address: satoruta@md.tsukuba.ac.jp

[4] Abbreviations used in this paper: Tc, cytotoxic T; CHS, contact hypersensitivity; DNFB, 2,4-dinitrofluorobenzene; LNC, lymph node cell; Tg, transgenic; WT mice, wild-type transgene-negative littermates.

Materials and Methods

Generation of T-bet Tg mice

A 2.5-kb, full-length cDNA encoding the murine T-bet protein was inserted into a VA CD2 transgene cassette containing the upstream gene regulatory region and locus control region of the human CD2 gene. The VA vector has been reported to direct expression of the inserted cDNA in all single-positive mature T lymphocytes of Tg mice, with expression being linearly proportional to the transgene copy number (19). This T-bet construct was injected into BDF1 fertilized eggs to generate Tg mice. T-bet Tg mice were inbred with C57BL/6 mice for four generations. Mice were maintained in specific pathogen-free conditions in a laboratory animal resource center. All experiments were performed according to the Guide for the Care and Use of Laboratory Animals at the University of Tsukuba (Ibaraki, Japan), and the study was approved by the Institutional Review Board of the university.

Southern hybridization analysis of genomic DNA

Southern hybridization was performed by using the Gene Images random prime labeling module system (Amersham Biosciences). High m.w. DNA was prepared from the tail of each mouse, and 15 μg of DNA was digested with *ApaI* and then subjected to electrophoresis on 1.0% agarose gels. After electrophoresis, the DNA was transferred to a Hybond-N^+ membrane. A fluorescence-labeled *ApaI/KpnI* fragment (0.5 kb) of the *T-bet* cDNA was used as a probe. The transgene copy number was determined from the blot with a BAS 1500 Mac image analyzer.

RT-PCR for transgene expression analysis

Total RNA was prepared from the thymus of 10 wk-old Tg mice or their wild-type transgene-negative littermates (WT mice) using TRIzol reagent according to the manufacturer's instructions (Invitrogen Life Technologies). First-strand cDNA was synthesized at 42°C for 50 min using the SuperScript II RNase H2 reverse-transcriptase kit (Invitrogen Life Technologies), and 1 μl of this 20 μl reaction mixture was used for the PCR. Amplified products were analyzed on 2% agarose gels. PCR primer sequences were as follows: T-bet, 5′-CGGTACCAGAGCGGCAAGT-3′ and 5′-AGCCCCCTTGTTGTTGGTG-3′; GATA-3, 5′-TCTCACTCTCGAG GCAGCATGA-3′ and 5′-GGTACCATCTCGCCGCCACAG-3′; GAPDH, 5′-CCCCTTCATTGACCTCAACTACATGG-3′ and 5′-GCCTGCTTCAC CACCTTCTTGATGTC-3′.

Western blot analysis

Thymocyte nuclear extracts were prepared from 10 wk-old Tg mice or WT. The extracts were size-fractionated on a 10% SDS-polyacrylamide gel, transferred to a polyvinylidene difluoride membrane (FluoroTrans), and reacted with primary and secondary Abs. For detection of the T-bet protein, a goat anti-mouse T-bet (N-19; Santa Cruz Biochemicals) was used as the primary Ab and peroxidase-conjugated rabbit anti-goat IgG (Zymed Laboratories) was used as the secondary Ab. For normalization with respect to the amount of protein in each sample, anti-lamin B Ab (Santa Cruz Biochemicals) was used as a control.

Histopathological analysis

Organs were fixed with 10% formalin in 0.01 M phosphate buffer (pH 7.2) and embedded in paraffin. Sections (3 μm) were stained with H&E for histopathological examination by light microscopy.

Measurement of serum Ig

Total serum Ig was determined by ELISA as previously described (20). Briefly, Nunc immunoplates were coated with goat anti-mouse Ig (ICN Pharmaceuticals). The plates were kept at room temperature for 1 h and then washed with 0.1 M PBS. After washing, the plates were blocked with 0.5% BSA in PBS solution. Serial dilutions of test serum samples were applied and incubated at room temperature for 1 h. After washing with PBS, the plates were treated with alkaline phosphatase-conjugated goat anti-mouse IgG, IgG1, or IgG2a (Sigma-Aldrich) at room temperature for 1 h. After additional washes, alkaline phosphatase substrate (Sigma-Aldrich) solution was added and allowed to develop. Absorption at 405 nm was measured with an immunoplate reader (BenchMark; Bio-Rad).

Culture medium, cytokines, and Abs

RPMI 1640 medium supplemented with 10% FCS, 2-ME (0.05 mM), L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), HEPES buffer (10 mM), and sodium pyruvate (1 mM) was used as culture medium. Recombinant mouse cytokines were IL-2 (Genzyme Techne), IL-4 (BD Pharmingen), and IL-12 (BD Pharmingen). Purified rat anti-mouse IL-4 (11B11), IL-12 (C17.8), CD3e (145–2C11), and CD28 (37.51) mAb, PE-conjugated anti-mouse IL-5 (TRFK5), and FITC-conjugated anti-mouse IFN-γ (XMG1.2) were purchased from BD Pharmingen.

Preparation of T Cells

$CD4^+$ and $CD8^+$ T cells were prepared from each mouse spleen and lymph nodes. $CD4^+$ and $CD8^+$ T cells were enriched by positive selection using a MACS system with anti-CD4 and anti-CD8 mAb (Miltenyi Biotec). In the spleen cell transfer experiment the cells (3×10^6 cells) were transferred i.v.

Stimulation of Tg $CD4^+CD8^+$ T cells for cytokine production

Primary stimulations of $CD4^+/CD8^+$ T cells (2.5×10^5 cells/well) were performed with cross-linked anti-CD3e (1 μg/ml) and anti-CD28 (10 μg/ml) plus IL-2 (10 ng/ml) in a total volume of 2 ml in 24-well plates. In addition, some cultures received cytokines (10 ng/ml IL-4 or 10 ng/ml IL-12) or mAb to block endogenous cytokines (10 μg/ml anti-IL-4 or 10 μg/ml anti-IL-12). T cells were expanded and maintained under constant culture conditions for 1 wk.

Flow cytometric analysis of intracellular IL-5 and IFN-γ synthesis

Cells were resuspended at 10^5 to 10^6 cells/ml and stimulated with PMA (50 ng/ml) plus ionomycin (500 ng/ml). Two hours before cell harvesting, brefeldin A was added at 10 μg/ml using a stock solution of 1 mg/ml in ethanol (100%). Cells were harvested, washed, and resuspended in PBS with brefeldin A before the addition of an equal volume of 4% formaldehyde fixative (final concentration, 2%). After fixation for 20 min at room temperature, cells were stained for cytokines. For intracellular staining, all reagents and washes contained 1% BSA and 0.5% saponin (Sigma-Aldrich), and all incubations were performed at room temperature. Cells were washed and preincubated for 10 min in PBS/BSA/saponin and then incubated with allophycocyanin-conjugated anti-mouse IL-5 (5 μg/ml) and anti-mouse IFN-γ (5 μg/ml) or isotype-matched control Abs (10 μg/ml) for 30 min. After 20 min, cells were washed twice with PBS/BSA/saponin and then washed with PBS/BSA without saponin to allow membrane closure. Samples were analyzed with a FACScalibur flow cytometer (BD Biosciences). Results were analyzed by using CellQuest software.

Induction of CHS

Induction of CHS was conducted using the methods described previously (21). Briefly, mice were sensitized to DNFB by painting the shaved abdomen with 50 μl of 0.5% DNFB in acetone/olive oil (4:1) and each footpad with 5 μl of the mixture on days 0 and 1. On day 5, mice were challenged with 20 μl of 0.3% DNFB on each side of the left ear. As a control, the right ear was painted with an identical amount of vehicle. The ear thickness was measured at 12, 24, 48, and 72 h after challenge at three locations. The ear swelling was calculated as $[(T - T_0)$ left ear$] - [(T - T_0)$ right ear], where T_0 and T represent the values of ear thickness before and after the challenge, respectively.

Results

Generation of Tg mouse lines overexpressing T-bet in T cells

To generate Tg mouse lines expressing high levels of T-bet specifically in T cells, the mouse *T-bet* cDNA was inserted into the VA vector (Fig. 1*A*). Genomic Southern blotting analysis was performed to confirm the integrity and copy number for each Tg mouse line. The length of the *ApaI* fragment containing the *T-bet* transgene was 1.2 kb, whereas the corresponding fragment for the endogenous *T-bet* gene was 4.0 kb (Fig. 1*A*). The transgene was detected in mice of Tg lines 710, 725, and 731 (Fig. 1*B*). In densitometric analyses, line 710 seemed to contain more than 12 copies of the transgene, whereas lines 725 and 731 contained approximately 12 and 8 copies, respectively. However, line 710 could not transmit the genes to the next generation, but the transgenes in both line 725 and line 731 were stably transmitted to progeny.

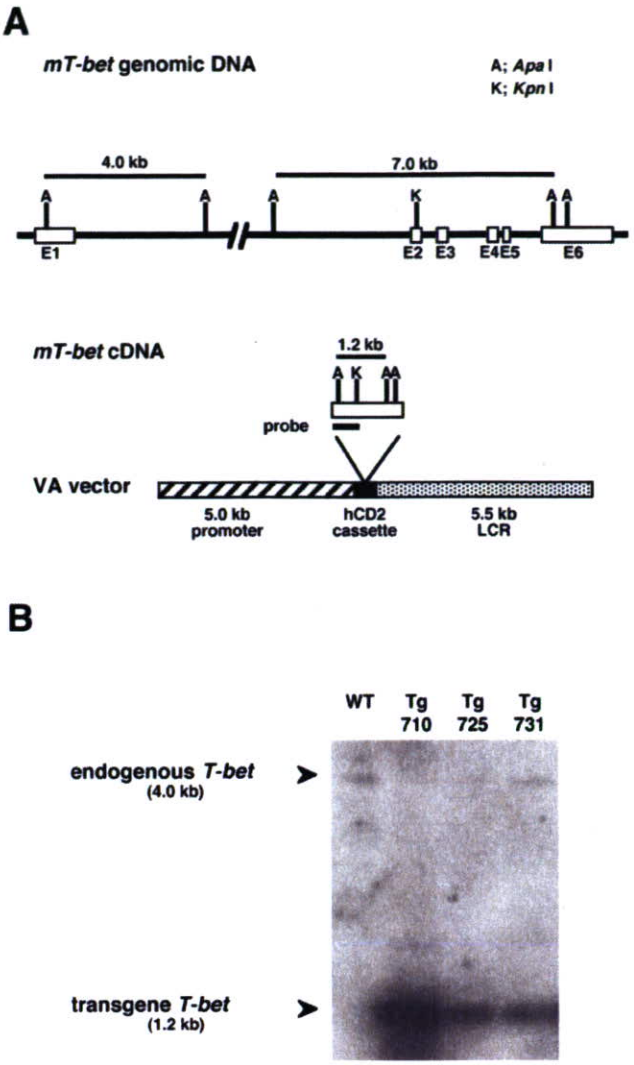

FIGURE 1. Generation of T-bet-overexpressing mice. *A*, Diagram showing the structures of the mouse *T-bet* (*mT-bet*) gene locus and the Tg construct. *T-bet* cDNA was inserted into a vector (VA vector) containing a human CD2 transgene cassette. The Southern blotting probe site, the restriction sites, and the predicted sizes of the endogenous gene and the transgene (with *Apa*I restriction sites) are indicated. E, Exon; LCR, locus control region. *B*, Southern blot analysis of the endogenous and Tg *T-bet* genes in Tg mice. The fragment with *Apa*I and *Kpn*I restriction sites in *mT-bet* cDNA in panel (*A*) was used as the probe. The 4.0-kb endogenous and 1.2-kb Tg genes are shown for Tg line 710 (Tg 710), Tg 725 (Tg 725), and Tg 731 (Tg 731) mice. The transgene copy numbers for Tg lines 710, 725, and 731 were over 12, 12, and 8 copies, respectively.

Overexpression of T-bet in Tg mice

To confirm expression of the transgene, RT-PCR and immunoblot analyses were performed to monitor T-bet mRNA and protein levels in thymocytes from the two Tg lines (Fig. 2, *A* and *B*). Overexpression of T-bet mRNA and protein was detected in all Tg mice tested. The amount of T-bet protein in Tg line 725 cells was slightly higher than the amount in Tg line 731, indicating that the expression level of the protein was copy number dependent. The T-bet protein was not detected in WT mice in this analysis.

Higher ratio between IgG2a and IgG1 in T-bet Tg mice

To determine cytokine levels we first analyzed serum by the ELISA method, but all samples were below the level of detection (data not shown). Because Th1/Th2 cytokines contribute to control of Ig subtype production, we next analyzed serum IgG1 and IgG2a. Th1 cells support macrophage activation, delayed-typed hypersensitivity responses, and Ig isotype switching to IgG2a. In contrast, Th2 cells provide efficient help for B cell activation and class switching to IgG1 (22, 23). To confirm the Th1-dominant response in T-bet Tg mice, serum IgG levels were measured by ELISA (Table I). Tg line 731 mice had serum total IgG levels similar to those of WT mice (Tg line 731, 394.0 ± 37.6 mg/dl; WT, 340.3 ± 18.7 mg/dl), but Tg line 725 levels were significantly higher than those of WT mice (547.4 ± 108.9 mg/dl). Serum IgG1 levels of Tg mice (Tg line 731, 79.4 ± 7.4 mg/dl) tended to be lower than those of WT mice (174.0 ± 33.7 mg/dl) but, in contrast, IgG2a levels were higher (Tg line 725, 253.1 ± 77.9 mg/dl; WT, 90.7 ± 12.6 mg/dl). To confirm the promotion of the IgG2a class switch and repression of IgG1 Tg mice, IgG2a/IgG1 ratios were calculated. These were found to be significantly higher in Tg mice (Tg line 725, 2.93 ± 0.95; Tg line 731, 1.22 ± 0.13) than in WT mice (0.62 ± 0.08) ($p < 0.01$).

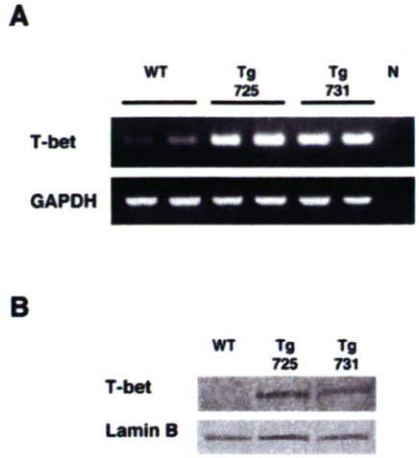

FIGURE 2. T-bet expression analysis by RT-PCR and Western blot in the thymus. *A*, RT-PCR analysis. In Tg line 725 (Tg 725) and Tg line 731 (Tg 731), T-bet gene expression was higher than that of WT. N, PCR without template as negative control. Two individual mice were used in each genotype. *B*, T-bet protein in nuclear extracts from thymocytes. T-bet protein was clearly identified by Western blotting in the extracts from Tg lines 725 and 731. In this analysis, however, the normal level of T-bet from WT thymocytes could not be detected.

Increased synthesis of IFN-γ in T-bet Tg mice

From the above data, Tg line 725 mice had greater overexpression of T-bet than Tg line 731 mice and were therefore used in the following studies. To confirm the observed differences in

Table I. *Serum immunoglobulins for 30-week-old mice*[a]

Measurement	WT (n = 12)	Tg Line 725 (n = 8)	Tg Line 731 (n = 10)
IgG (mg/dl)	340.3 ± 18.7	547.4 ± 108.9[b]	394.0 ± 37.6
IgG1 (mg/dl)	174.0 ± 33.7	174.9 ± 89.0	79.4 ± 7.4[b]
IgG2a (mg/dl)	90.7 ± 12.6	253.1 ± 77.9[b]	97.1 ± 13.4
IgG2a/IgG1 ratio	0.62 ± 0.08	2.93 ± 0.95[c]	1.22 ± 0.13[c]

[a] Data are expressed as mean ± SEM.
[b] $p < 0.05$ versus WT.
[c] $p < 0.01$ versus WT.

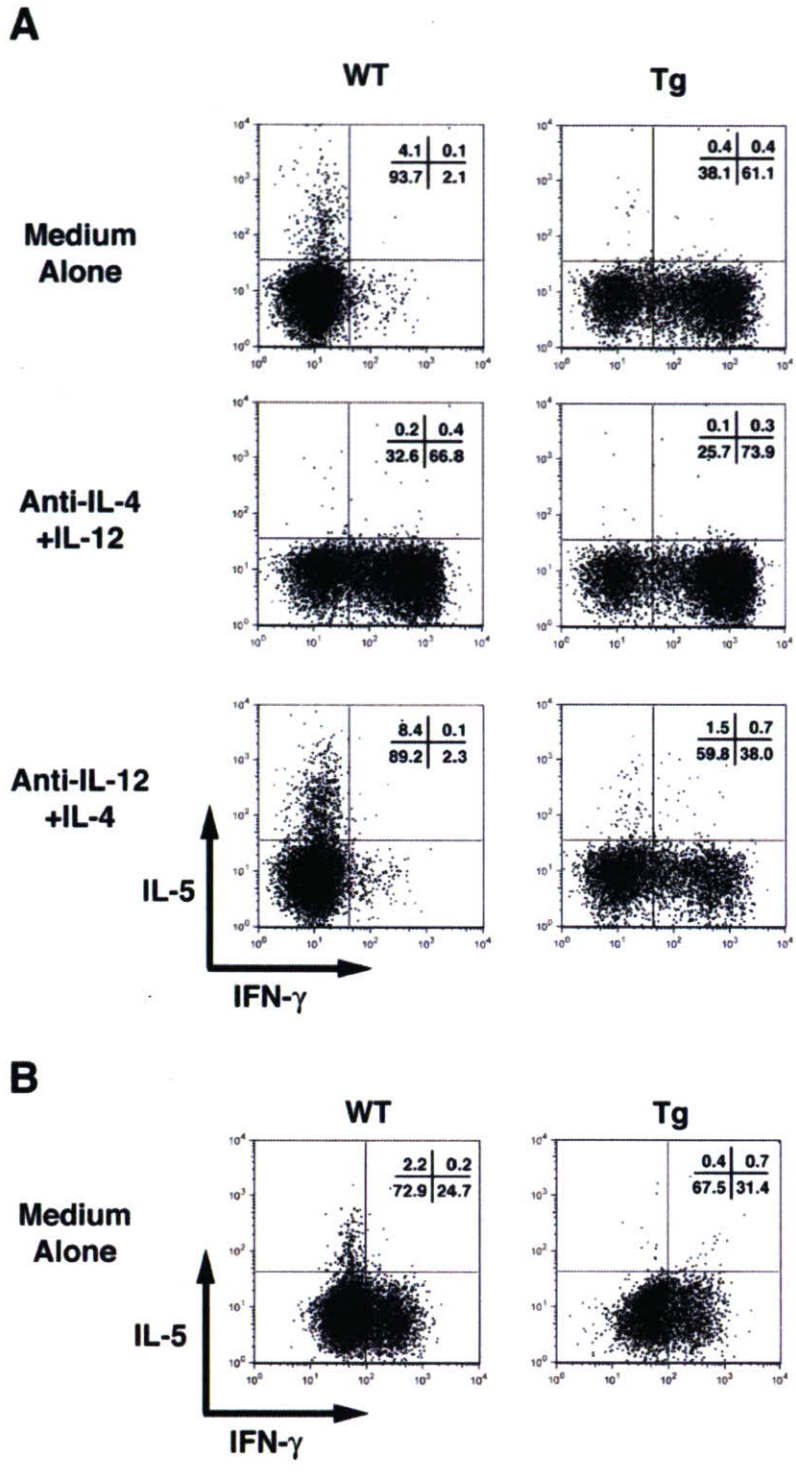

FIGURE 3. Intracellular cytokine analysis of $CD4^+$ (*A*) and $CD8^+$ (*B*) T cells from each group. *A*, $CD4^+$ T cells from WT and Tg line 725 mice were cultured in the presence of medium alone, anti-IL-4 plus IL-12 (Th1 differentiation conditions), or anti-IL-12 plus IL-4 (Th2 differentiation conditions) and analyzed by flow cytometry for intracellular synthesis of IFN-γ and IL-5. The frequencies of IFN-γ-producing cells are shown on the *x*-axis and those of IL-5-producing cells on the *y*-axis. Intracellular synthesis of IFN-γ in Tg line 725 mice was increased under all conditions. *B*, $CD8^+$ T cells from WT and Tg line 725 mice were cultured in the presence of medium alone and analyzed by flow cytometry for intracellular synthesis of IFN-γ and IL-5. Results are representative of three independent experiments.

cytokine production at the single-cell level, we studied their intracellular synthesis by flow cytometry. $CD4^+$ T cells from Tg mice had higher levels of IFN-γ than WT mice either in medium alone or under conditions favoring Th1 differentiation (presence of anti-IL-4 Ab and IL-12) or Th2 differentiation (presence of anti-IL-12 Ab and IL-4) (Fig. 3*A*). Especially in the Th2 condition 38.0% of $CD4^+$ T cells from Tg mice produced IFN-γ, but only 2.3% from WT did so. In contrast to IFN-γ, the production of IL-5 in cells from Tg mice showed lower levels in medium and the Th2 condition. IL-4 production

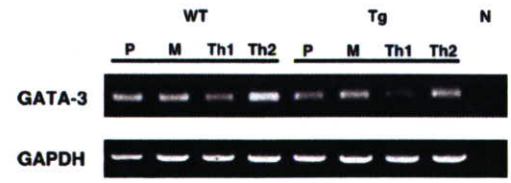

FIGURE 4. GATA-3 mRNA expression analysis by RT-PCR in intracellular cytokine production experiment. P, Prestimulation; M, medium-alone condition; Th1, Th1 differentiation conditions; Th2, Th2 differentiation conditions; N, PCR without template as negative control.

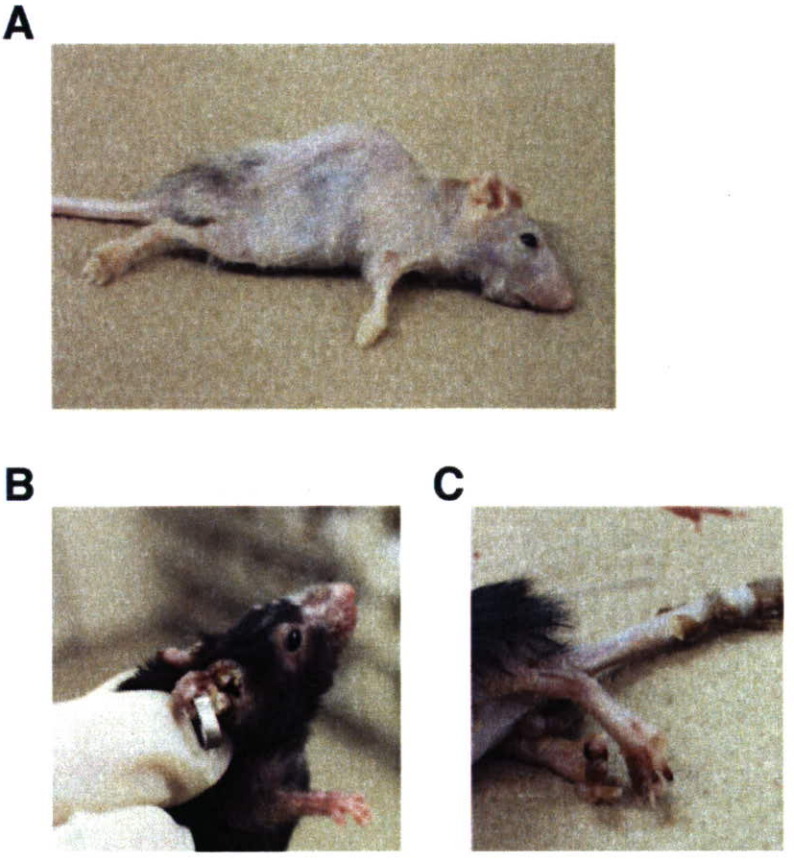

FIGURE 5. Tg mice develop dermatitis. In severe cases, individual Tg mice lost hair all over the body (*A*). In mild cases, the surface of the face, ear, foot, and tail showed redness and scaling (*B* and *C*).

analyses were also done. IL-4 production in cells from Tg and WT mice was similar and the percentage of positive cells was very low under all conditions (data not shown). These results demonstrate that T cells from T-bet Tg mice have a dominant Th1 differentiation pattern and suggest that overexpression of T-bet prevents Th2 differentiation. $CD8^+$ T cells from Tg mice also had higher levels of IFN-γ than WT mice in neutral condition, but not so markedly as that of $CD4^+$ T cells from Tg mice (Fig. 3*B*). We also determined the GATA-3 mRNA expression in the intracellular cytokine production analyses (Fig. 4). GATA-3 mRNA expression in Tg mice showed lower levels than those of wild mice, especially in a Th1 condition.

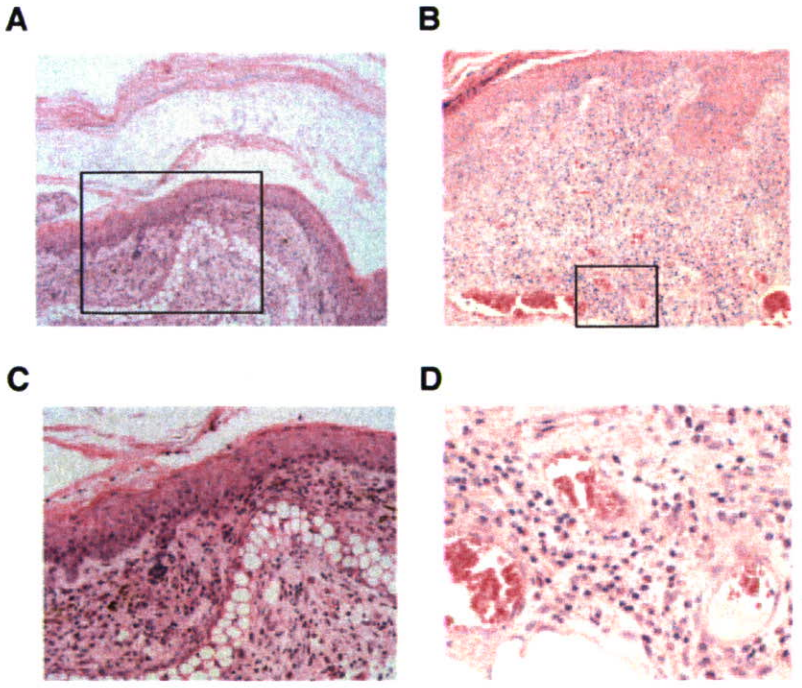

FIGURE 6. Histological appearance of the ear skin. *A* and *B*, In the histological analysis of ear skin, hyperkeratosis, acanthosis, broadening of the papillae, and infiltration of neutrophils lymphocytes, and melanophages are seen. *C and D*, At higher magnification of the squares from *A* and *B*, infiltration of mononuclear cells and neutrophils is observed. (H&E staining; magnification: ×100 (*A*), ×100 (*B*), ×200 (*C*), and ×400 (*D*).

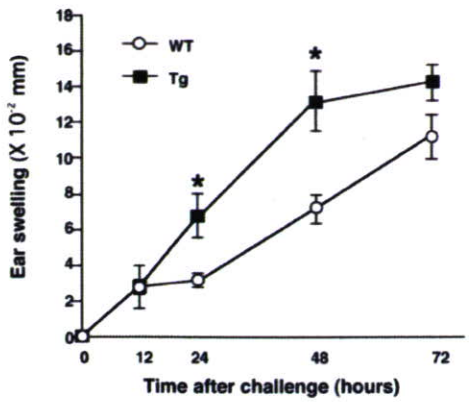

FIGURE 7. CHS reaction to DNFB in Tg and WT mice. The CHS response was determined by ear swelling at various times after hapten challenge. Time after challenge was 24 and 48 h; the ear swelling values in Tg mice (■, $n = 5$) were significantly higher than those in WT mice (○, $n = 5$). *, $p < 0.05$.

Development of contact dermatitis in T-bet Tg mice

During the initial analysis of the Tg cohorts we found that they developed dermatitis. At ~10 wk of age, ~28% of Tg line 725 (12 of 43) and 5% of Tg line 731 (3 of 61) mice spontaneously developed dermatitis characterized by swollen, flaky, and scaly skin in regions lacking body hair (e.g., tail or ears), which in some individuals progressed all over the body, together with alopecia (Fig. 5). Histological examination of the affected skin showed epidermal hyperkeratosis and neutrophil and lymphocyte infiltration similar to what is seen in contact dermatitis in humans (Fig. 6). To prove that the skin lesion was contact dermatitis although the Ag was not determined, we performed cell transfer experiment. We transferred total spleen cells from T-bet to WT mice and found that dermatitis was induced in 70% (7 of 10 mice) within one month. However, dermatitis was not observed after the transfer of separated $CD4^+$ or $CD8^+$ cells alone.

Augmentation of CHS reactions in Tg mice

To determine the role of T-bet overexpression in T cell subpopulations in CHS responses, 6-wk-old WT and Tg (Tg line 725) mice, which did not develop dermatitis, were sensitized with DNFB as described in *Materials and Methods*. Ear swelling responses to DNFB were significantly increased in Tg mice compared with WT mice (Fig. 7). The CHS response was significantly higher at 24 and 48 h after challenge. Histological analysis of hapten-treated WT and Tg ears showed characteristic features of CHS including dermal edema, mononuclear cell infiltration, and vascular enlargement (Fig. 8*B*). These histological changes were dramatically enhanced in hapten-treated Tg mouse ears (Fig. 8*B*).

Both $CD4^+$ and $CD8^+$ T cell lymph node cells (LNCs) produce significant amounts of IFN-γ in T-bet Tg mice

To determine IFN-γ production in skin-draining lymph nodes of Tg and WT mice, DNFB-primed LNCs were cultured under condition with medium alone. A significant increase of IFN-γ-producing cells was found among both $CD4^+$ and $CD8^+$ LNCs from DNFB-stimulated Tg mice (Fig. 9). In Tg mice, IFN-γ-producing cells were increased in $CD4^+$ LNCs even without DNFB stimulation. After stimulation, IFN-γ-producing cells increased further (Fig. 9*A*). Regarding $CD8^+$ LNCs, WT and Tg mice had the similar levels of IFN-γ-producing cells, but after DNFB stimulation the increase of IFN-γ-producing cells was greater in Tg mice (Fig. 9*B*). IL-10 production was also analyzed as a marker cytokine of

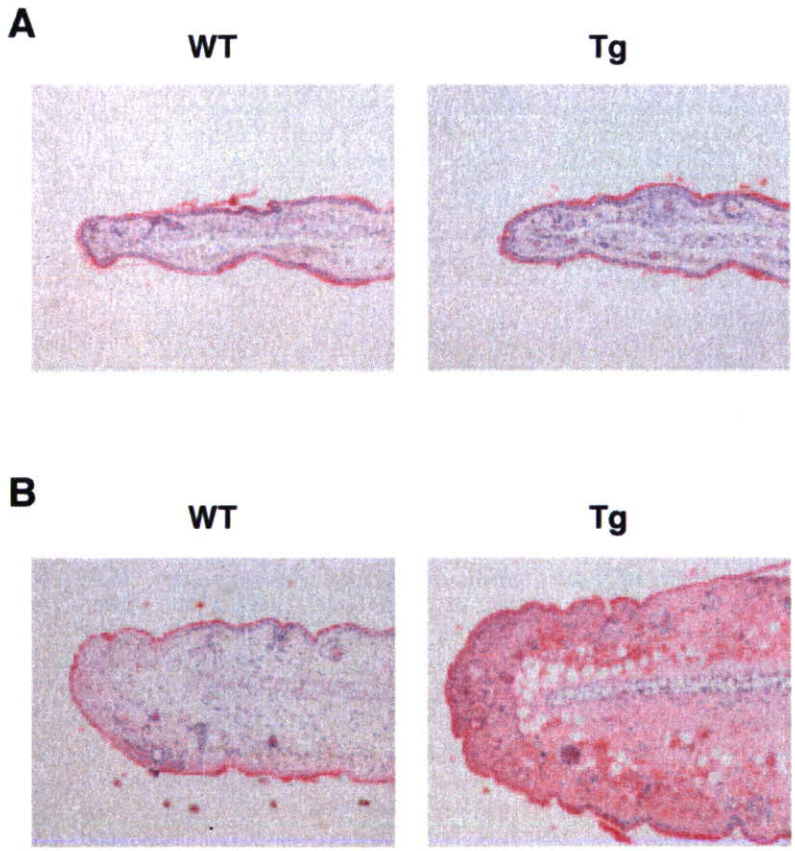

FIGURE 8. Representative results from histological analysis of CHS reactions. At 72 h after challenge, skin sections are shown from vehicle-treated mice (*A*) and hapten-treated mice (*B*) (H&E staining; magnification: ×100). *A*, Sections from vehicle-treated Tg mice exhibited normal histological features similar to those of vehicle-treated WT mice. *B*, Sections from hapten-treated WT and Tg mice displayed characteristic features of the CHS reaction including dermal edema, mononuclear cell infiltration, and vascular enlargement (*B*). The severity of the CHS reaction was greater in Tg mice.

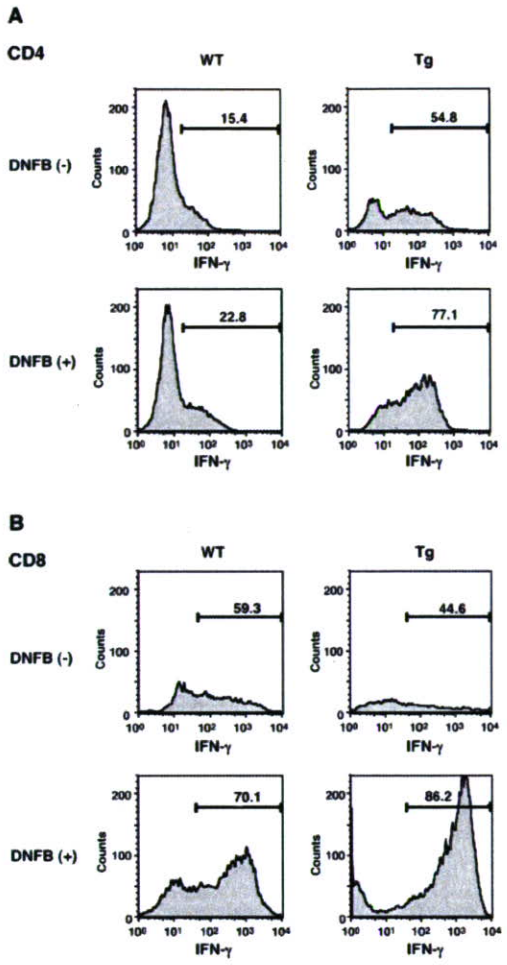

FIGURE 9. The number of IFN-γ-producing cells among $CD4^+$ and $CD8^+$ T cells was increased in CHS-sensitized Tg LNCs. LNCs from 72 h DNFB-sensitized or non-sensitized WT and Tg mice were cultured in medium alone and subjected to intracellular cytokine staining as described in *Materials and Methods*. Significantly higher numbers of IFN-γ-producing cells were detected in DNFB-sensitized Tg $CD4^+$ (*A*) and $CD8^+$ (*B*) T cells. Results are representative of three independent experiments.

Th2 or Tc2. However, no significant differences between Tg and WT mice were found (data not shown).

Discussion

T-bet is known as a master regulator of Th1 development. It induces IFN-γ production and repression of Th2 cytokines in vitro (7). In this study, we generated T-bet-overexpressing mice characterized by Th1-dominant responses in vivo and in vitro. Thus, T-bet Tg mice showed Th1-dominant Ig production in vivo and Th1-dominant intracellular cytokine production in vitro. Because serum cytokines were below the level of detection, we analyzed serum Ig isotypes. IFN-γ promotes IgG2a class switching while IL-4 promotes IgG1 class switching, so concentrations of serum IgG2a and IgG1 reflect Th1 and Th2 responses in vivo (22–24). The higher IgG2a/IgG1 ratio present in the sera of Tg mice suggests that T-bet overexpression promotes IgG2a class switching and represses IgG1 class switching. At the same time, we analyzed intracellular cytokines and found that $CD4^+$ T cells from Tg mice produced higher levels of IFN-γ than WT mice under neutral, Th1- or Th2-promoting conditions. In contrast, IL-5 production in $CD4^+$ T cells from Tg mice was lower than that in $CD4^+$ T cells from WT mice under neutral or Th2-promoting conditions. The serological Ig findings are therefore consistent with data from $CD4^+$ T cell intracellular cytokine assays, emphasizing that these do indeed reflect the Th1/Th2 balance in vivo. T-bet has also been shown to regulate cytolytic effector mechanisms of $CD8^+$ T cells (8). Its expression is rapidly induced in $CD8^+$ T cells by signaling through the TCR and the IFN-γR, and it functions downstream of STAT1 (6, 9, 10). In the context of Ag-specific activation, T-bet is required for the differentiation of naive $CD8^+$ T cells into effector CTLs. In the present study, Tg mice also had a higher fraction of IFN-γ-producing $CD8^+$ T cells according to intracellular cytokine assays, but not as markedly as $CD4^+$ T cells. However, Tg mice showed higher response for IFN-γ-producing $CD8^+$ T cells in the CHS response. From the above results, we speculated that Tg mice not only had a Th1-dominant background but also a potential Tc1-dominant background.

In this study, spontaneous skin inflammation was observed in Tg mice, first occurring in regions lacking body hair such as the tail or ears, where the skin is easily in contact with external agents. Histological examination of affected skin showed epidermal hyperkeratosis, spongiosis, and neutrophil and lymphocyte infiltration. This is typical for contact dermatitis, a delayed-type hypersensitivity reaction. It is known that Th1 activity greatly contributes to the development of dermatitis (25, 26). It is shown here that a Th1-dominant response occurs in T-bet Tg mice. Tg mice expressing IFN-γ in the epidermis have also been investigated previously and found to have reddened skin, growth retardation, hair loss, and flaky skin lesions (25). Keratinocyte proliferation was increased and there was epidermal thickening with spongiosis and parakeratosis. The possible importance of several cytokines such as IL-2 (27), IL-6 (28), and IL-7 (29) in the development of contact dermatitis has also been investigated using Tg mouse models (30). In this study we used Tg mice overexpressing the T cell differentiation transcription factor T-bet, but not cytokine transgenes, to address contact dermatitis for the first time. The results from this study suggested that transcriptional regulation of T-bet might play an important role in contact dermatitis.

Contact allergens such as DNFB, oxazolone, and 2,4-dinitrochlorobenzene are used to induce CHS. In the present study we also showed that Tg mice responded significantly to DNFB. There have been major controversies on the respective roles of $CD4^+$ and $CD8^+$ T cells in the development of CHS inflammatory reactions (12). However, it has become clear that both $CD4^+$ and $CD8^+$ T cells can act as effector cells in this context (17). Wang et al. (17) showed that both $CD4^+$ Th1 and $CD8^+$ Tc1 cells are crucial effectors in the CHS response to DNFB. Previous studies have also demonstrated that cytokines play important effector and regulatory roles in CHS responses. The type 1 cytokine IFN-γ promotes CHS, whereas type 2 cytokines down-regulate CHS responses (31–33). It has been demonstrated that type 1 cytokines can be produced by $CD8^+$ Tc1 cells as well as by $CD4^+$ Th1 cells in the same way that type 2 cytokines can be derived from Th2 and Tc2 cells (5, 34). Intracellular cytokine analyses showed that T-bet-overexpressing Tg mice had a large fraction of IFN-γ-positive cells within both $CD4^+$ and $CD8^+$ T cells. We also transferred total spleen cells from T-bet to WT mice and found that dermatitis was induced in 70% (7 of 10 mice) within 1 mo. However, dermatitis was not observed after the transfer of separated $CD4^+$ or $CD8^+$ cells alone. These results suggest that both $CD4^+$ Th1 and $CD8^+$ Tc1 cells may be active as effector cells in CHS responses in this model. Furthermore, it is known that activated cells produce IFN-γ to activate skin resident cells, which produce cytokines and chemokines allowing the recruitment of the polymorphonuclear cell infiltrates characteristic of CHS. This effector phase of CHS takes

24 to 48 h in the mouse (12), coinciding closely with our results on 24- and 48-h DNFB hyperresponsiveness.

In contrast to T-bet, GATA-3 is known as a Th2 lineage commitment transcription factor (35, 36). We have previously shown that GATA-3 overexpression in Th1-dominant autoimmune disease can diminish autoimmune nephritis (20, 37) and, therefore, that therapy to regulate expression levels of transcriptional factors may be useful to control unbalanced Th1/Th2 activity in many diseases. The results of the present study also suggest that to control Th1 and Tc1 reactions via down-regulation of T-bet expression might be useful for alleviating contact dermatitis.

In conclusion, we have generated Th1- and Tc1-dominant mice that developed spontaneous skin inflammation very similar to contact dermatitis. These mice should be useful for revealing a link between some immune diseases and Th1/Th2 and Tc1/Tc2 dysbalance and may offer a valuable murine model of contact dermatitis.

Acknowledgment

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Disclosures

The authors have no financial conflict of interest.

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Expression of Syk is associated with nasal polyp in patients with allergic rhinitis

Yuki Hamajima [a,*], Sigeharu Fujieda [b], Hiroshi Sunaga [b], Takechiyo Yamada [b], Kazuho Moribe [c], Nobuhiro Watanabe [a], Shingo Murakami [a]

[a] *Department of Otorhinolaryngology, Nagoya City University, 1, Kawasumi, Mizuho-ku, Nagoya 467-8601, Aichi, Japan*
[b] *Department of Otorhinolaryngology, Head & Neck Surgery, University of Fukui, Matsuoka, Fukui, Japan*
[c] *Department of Otolaryngology, Ichinomiya Municipal Hospital, Japan*

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Abstract

Objective: Numerous signalings are involved in allergic inflammation. The non-receptor protein tyrosine kinase, Syk, is widely expressed in immune-potentiated cells and plays critical roles in initiating signal transduction in response to the activation of cytokine, chemokine and other types of receptors. It has been hypothesized that Syk expression in allergic nasal mucosa and polyps with allergy is different from non-allergic mucosa, and that changes in Syk expression contribute to the activation of allergic reactions.
Methods: We examined whether the expression of Syk is found in allergic nasal mucosa and polyps. We investigated the expression of Syk in 46 nasal mucosa and polyps (14 samples from patients with allergic rhinitis and 32 samples with non-allergic chronic sinusitis) using an immunohistochemical technique.
Results: Allergic polyps had more Syk positive cells than non-allergic polyps. Syk positive cells were determined to mainly be eosinophils. There was no difference in Syk expression in the lamina propria and nasal gland between allergic mucosa and non-allergic mucosa.
Conclusion: Eosinophils in allergic polyps receive an intracellular signal, although the signal is not able to determine the function in the present state. Syk appears to be a promising target molecule for anti-allergic inflammation in allergic rhinitis.

Keywords: Nasal polyp; Syk; Eosinophil; Mast cell; Nasal allergy

1. Introduction

Nasal polyps are recurrent protrusions of the nasal sinus mucosa that prolapses into the nasal cavity. The origin of nasal polyps can be divided into two categories: allergic rhinitis (AR) and non-allergic chronic sinusitis (NACS). In AR, exposure to the allergen promotes the cross-linking of IgE molecule on mast cells and mast cells release histamine and other proinflammatory mediators within minutes. The released chemical mediators induce many signals in the cells of the nose and the sum of signals causes allergic inflammation. Chemoattractants play a dual role by triggering integrin activation and directing leukocyte migration. Some chemokines and cytokines (RANTES, Eotaxin, IL-5, etc.) have been shown to attact and activate eosinophils *in vitro* and to recruit eosinophils into the inflammatory region in the nasal mucosa. The addition of several factors (ex. edema, a change in architecture of the epithelium, a large influx of water, an alteration of the structure of gland) to the AR contributes to the development of nasal polyp [1].

NACS may originate from or be perpetuated by local or systemic factors that predispose one to sinus ostial obstruction and infection [2]. Japanese NACS is different from Western NACS, and has a characteristic that neutrophils are the predominant infiltrating cells in nasal mucosa [3]. A high level of IL-8 concentration in the nasal lavage from patients with nasal polyps is typical in Japanese NACS [3–5]. Persistent inflammation due to bacterial

* Corresponding author. Tel.: +81 52 853 8256; fax: +81 52 851 5300.
E-mail address: hamajima@med.nagoya-cu.ac.jp (Y. Hamajima).

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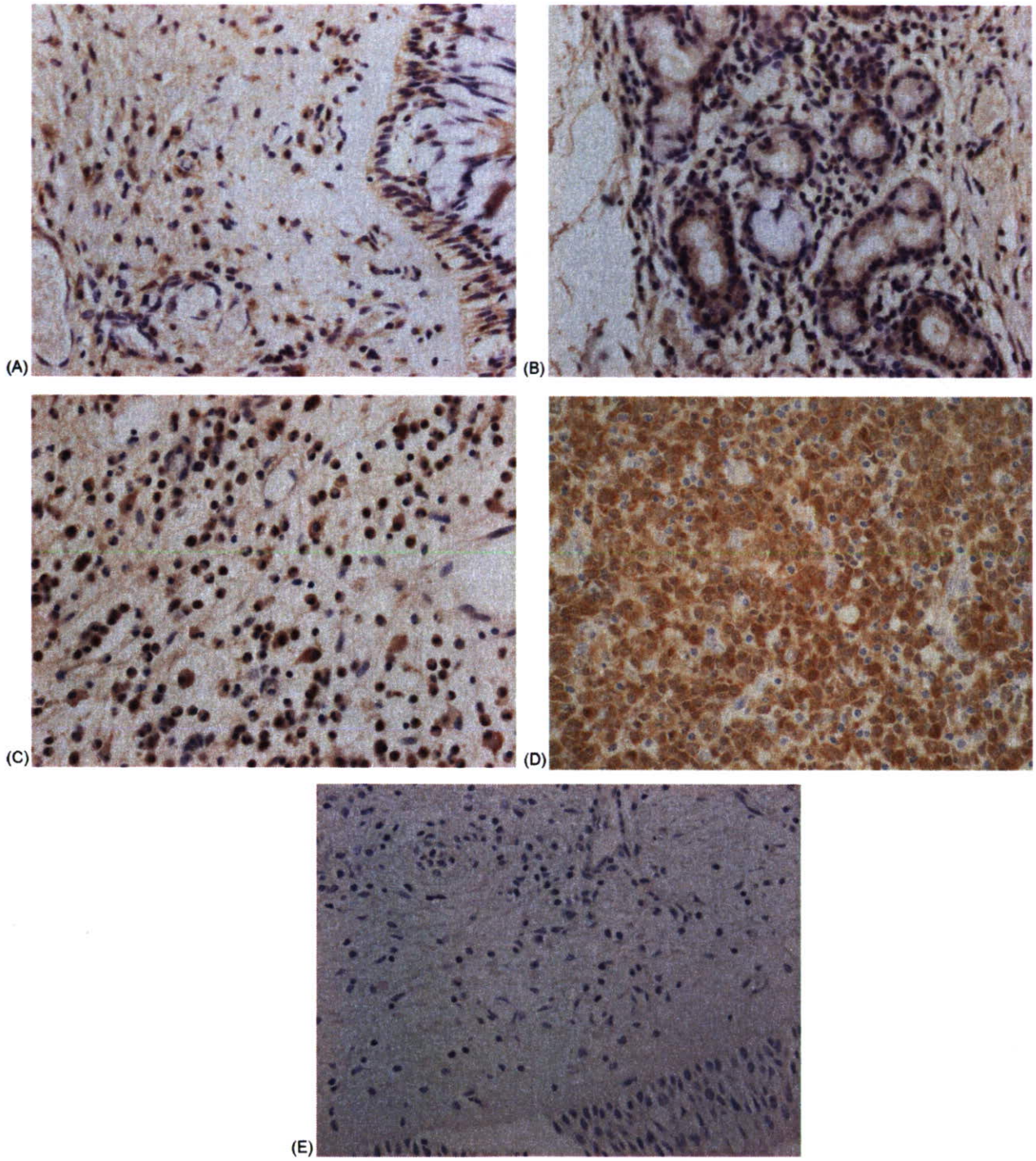

Fig. 1. Immunohistchemical staining of Syk in the nasal mucosae, intestinum of lamina propria (a: Syk score = 29.5), the nasal grand (b: Syk score = 46.5), nasal polyp (c: Syk score = 57.5), the tonsil was for the positive staining of Syk (d: Syk score = 48.8) nasal polyp (e: negative control) (magnification 200×).

infection and sequential cytokine secretion (IL-1β, TNFα, IL-8, TGFα and TGFβ) induced many signals in inflammatory cells, fibroblasts and epithelial cells [6,7]. The activation of epidermal growth factor receptor kinase and platelet-derived growth factor receptor kinase leads to hyperplasia of epithelial cells and nasal glands for the pathogenesis of nasal polyps in NACS [8,9].

The complicated activation in cells through cytokine and chemokine receptors is associated with protein tyrosine kinase. Protein tyrosine kinases can be divided into two

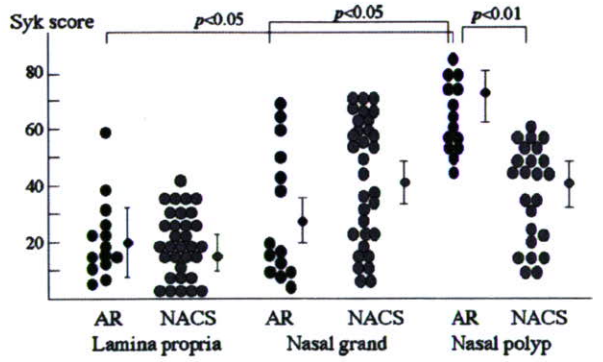

Fig. 2. The number of Syk positive cells. The Syk score determined the number of Syk positive cells in all cells. The Syk score in the nasal polyp with allergic rhinitis (AR) was higher than those with non-allergic chronic sinusitis (NACS).

families: receptor tyrosine kinases and non-receptor tyrosine kinases. Syk has non-receptor protein tyrosine kinases function as critical components in signaling cascades from membrane receptors lacking intrinsic tyrosine kinase activity, and is widely expressed on hematopoietic cells: B cells [10,11], mast cells [12], eosinophils [13], T cells [14], neutrophils [15] and other cells. Recently, it has been shown that Syk is also expressed in non-hematopoietic cells; human nasal fibroblasts [16,17], breast epithelium [18] and human hepatocytes [19]. The level of Syk expression in nasal polyp-derived fibroblasts was correlated with RANTES production by LPS [16].

In this study, we investigated the Syk expression on nasal polyps between AR and NACS using an immunohistochemical technique, since there are few reports about the expression of signal molecules in nasal polyps.

2. Materials and methods

2.1. Patients and sample collections

We studied the inferior turbinate mucosa and nasal polyps of 14 patients (10 males and 4 females) with perennial AR caused by *Dermatophagoides Pteronyssinus* (DP), and 32 control subjects (20 males and 12 females) with NACS. All of the patients underwent endoscopic sinus surgery, because all conservative treatments had no effect on their nasal congestion. We obtained informed consent from all patients. Both the inferior turbinate mucosa and the nasal polyps were excised during surgery. All of the patients with AR had a high titer of anti-DP-specific IgE without Japanese Cider and Ragweed in the serum. All patients with NACS had negative evidence for these allergies. No patients in either group had histories of aspirin-induced asthma. There were no significant differences in the background of the patients in AR and NACS groups except for nasal allergy. The operation was performed under local anesthesia by injection with 0.5% lidocaine with 1:100,000 adrenalin and 10% cocaine.

2.2. Immunohistochemical staining

Specimens were immediately fixed in 4% paraformaldehyde for over 48 h. After fixation, samples were embedded in paraffin and sectioned at 2–4 μm thickness. They were deparaffinated and treated with ethanol, then rinsed with pH7.4 phosphate-buffered saline (PBS). Immunohistchemical staining was performed using the avidin–biotin-complex technique [20].

Specimens were washed in distilled water, and rinsed with pH 7.4 PBS, and incubated in 0.3% H_2O_2 solution dissolved in absolute methanol at room temperature for 15 min to inhibit endogenous peroxidase activity. After washing, specimens that would be stained for Syk were treated by microwave irradiation for 10 min in distilled water. They were rinsed with PBS and incubated with rabbit anti-human Syk polyclonal antibodies diluted at 1:200 at 4 °C for 24 h. After rinsing with PBS, all specimens were treated with polymerized peroxidase anti rabbit-IgG (DAKO, Glostrup, Denmark) for 1 h at room temperature. After rinsing with PBS, peroxidase color visualization was carried out with 15 mg of 3–3′-diaminobenzidine tetrahydrochroride (WAKO, Tokyo, Japan) dissolved in 100 ml PBS with 8 μl of 30% H_2O_2 for 10 min. Nuclear counter staining was carried out with Mayer's haematoxylin for 2 min before mounting. For positive controls, we used the tissue of the tonsil, which was already known to be positive for Syk. For the negative control, we used rabbit anti-human IgG for the first antibody.

2.3. Double immunofluorescence techniques

The standard double immunofluorescence technique was employed. Mouse anti-human EG2 monoclonal antibody (mAb) as a marker of eosinophils, anti-CD30 mAb for T cells, anti-CD14 mAb for macrophages, anti-elastase mAb for neutrophils, anti-tryptase for mast cells, and anti-CD20 mAb for B cells were used. After being incubated with anti-human Syk antibody, rinsing with tris buffered saline (TBS), we applied swine FITC conjugated anti rabbit immunoglobulins for 2 h at room temperature. After rinsing with TBS, we applied the second antibody (for example: CD20) at 4 °C for 24 h. After rinsing with TBS, we treated rabbit RPE conjugated anti-mouse immunoglobulin for 2 h at room temperature. After washing with TBS, nuclear counter staining was carried out with Mayer's haematoxylin for 2 min before mounting. Then, we counted the positive cells with fluorescence microscopy. In this experiment, 7 NACS samples were randomly selected.

2.4. Evaluation of Syk expression

For microscopic analysis, we randomly selected five images of strongly stained sections of Syk in each specimen. The mean number of Syk positive cells per field, that had infiltrated the intestinum of the lamina propria, goblet cell,

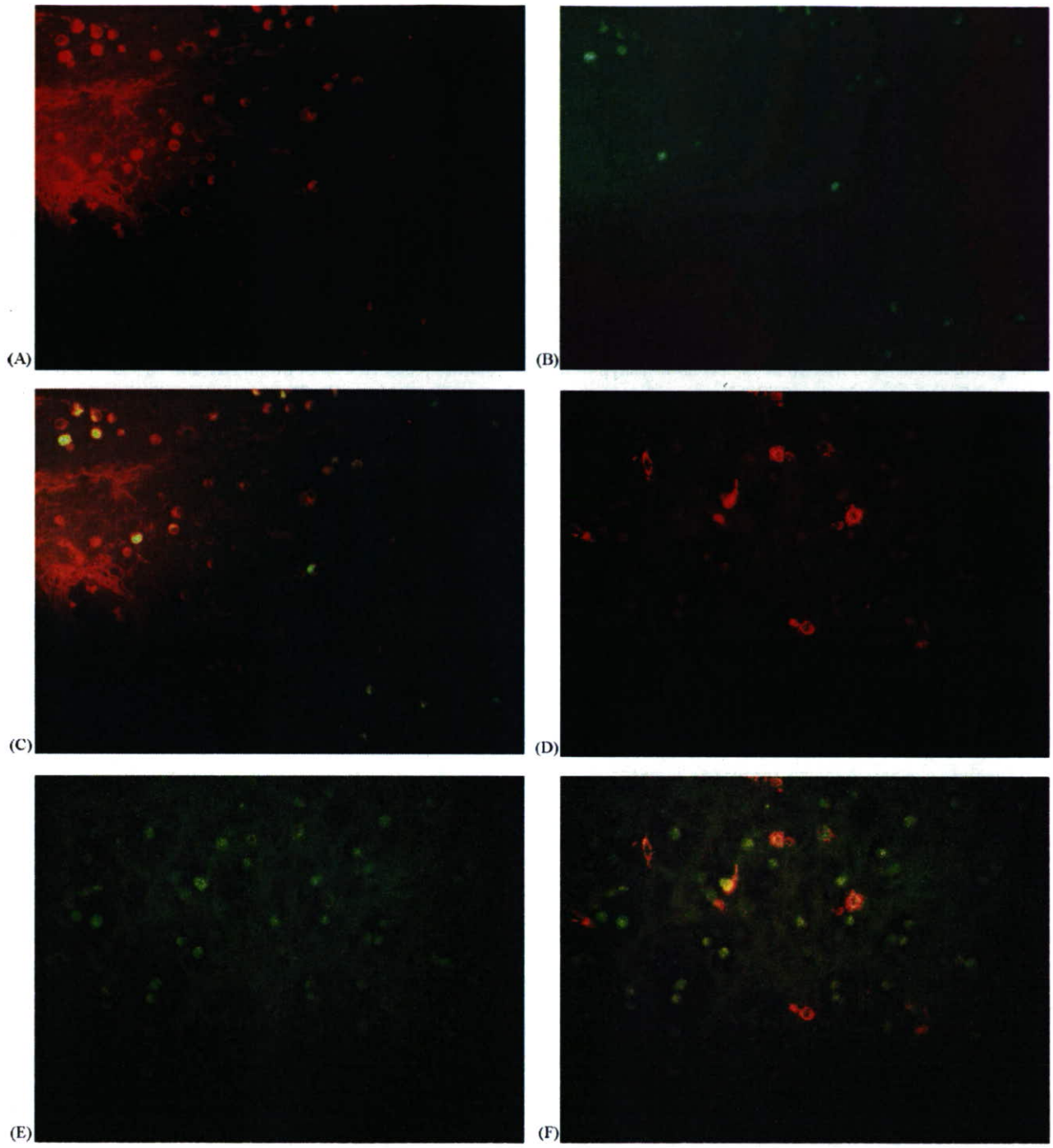

Fig. 3. Double immunohistchemical staining. (A) EG2 positive cells (red); (B) Syk positive cells (green); (C) merged view Syk score is 0.44 in this section (magnification 200×); (D) mast cell positive cells (red); (E) Syk positive cells (green); (F) merged view Syk score is 0.16 in this section (magnification 200×) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.).

epithelial layer were counted. We counted at least 1000 cells, including Syk positive cells, and estimated the positive cells as the Syk score [20].

For fluorescence analysis, we used a microscope (BX51-33-FL-3, Olympus, Tokyo, Japan). We randomly selected three images of strongly stained sections of Syk. The mean number of Syk and other per field that had infiltrated the nasal polyp were counted. These analyse were performed at a magnification of 400× (Syk positive cells). Macintosh computers (Stat view software; Abacus Concepts Inc., Berkeley, CA) were used for all statistical analysis.

3. Results

3.1. Syk expression in the nasal mucosa and polyp

Syk positive cells were observed in the lamina propria, nasal gland and nasal polyp. Syk staining was positive in the cells that had infiltrated the lamina propria and the nasal gland (Fig. 1A and B). The usual pattern of positive staining for Syk involved the cytoplasm. The mean Syk positive cells per field (Syk score) in the intestinum of lamina propria from all 46 patients was 15.9 ± 11.4 (mean ± S.D.), that in the nasal gland was 32.2 ± 22.4, and that in nasal polyp was 38.0 ± 19.0. In the AR group, the mean Syk score in the intestinum of lamina propria was 18.0 ± 11.7, that in the nasal gland was 25.8 ± 21.1, that in the nasal polyp was 50.9 ± 17.9. Syk score in the nasal polyp was higher than that in the lamina propria and nasal gland ($p < 0.05$, $p < 0.05$). In the NACS group, the mean of the Syk score in the intestinum of the lamina propria was 14.8 ± 11.2, that in the nasal grand was 35.3 ± 22.6, that in the nasal polyp was 30.2 ± 15.2 (no difference among the NACS group). In the AR group, the Syk score in the nasal polyp was significantly higher than that in the NACS group (50.9 ± 17.9 versus 30.2 ± 15.2, $p < 0.01$, Fig. 2). Few Syk positive cells were detected in the epithelial layer (data not shown).

3.2. Double staining

To clarify which cells expressed Syk in the nasal polyp from patients with AR, double immunostaining was performed. EG2 and Syk double positive cells were mainly observed in the allergic polyp (Fig. 3). In the AR group, the mean percentage of double positive staining for Syk and EG2 was higher than the NACS group (29.4 ± 21.4% versus 11.5 ± 8.7%, $p < 0.05$, Fig. 4). Since the Syk score of the nasal polyp in AR was higher than that in the NACS group, the absolute number of double positive nasal polyp cells in AR was also higher than that in NACS. Double positive cells

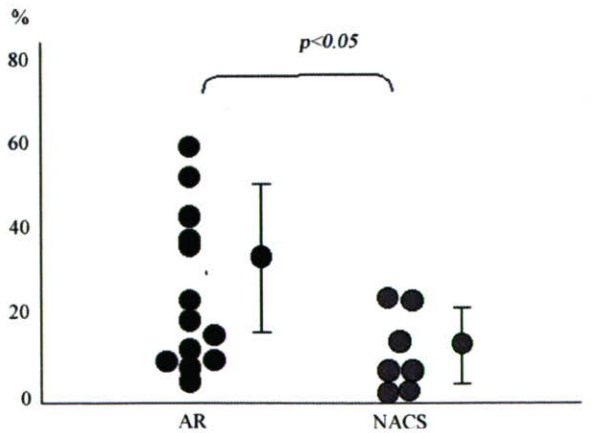

Fig. 4. The mean percentages of EG2 and Syk double positive cells. The double positive cells in AR weres higher than those in NACS ($p < 0.05$).

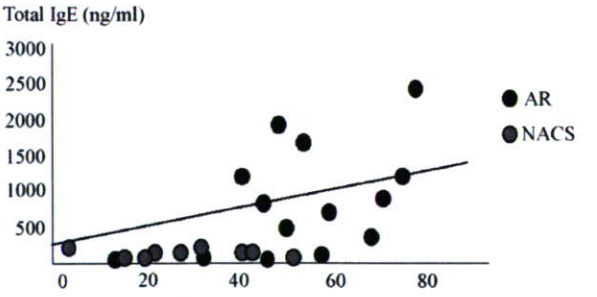

Fig. 5. The correlation with serum IgE titer and Syk score in AR. Total IgE in the serum was correlated with Syk score of nasal polyp from AR patients.

for Syk with each CD30 or elastase or CD14 were only minimally detected in both the AR and NACS group (data not shown). Although CD20 positive cells were stained in AR, the mean percentage of double positive cells in the CD20 cells was 1.1 ± 0.9%. And the mean percentage of double positive cells in the tryptase positive cells was 10.92 ± 16.85%.

3.3. Correlation of Syk score and IgE in the serum

Serum total IgE values in AR range from 16 to 2400, while those values in NACS range from 8 to 130. Total IgE in the sera is significantly associated with the Syk score in the nasal polyp ($r = 0.640$, $p < 0.01$, Fig. 5). There is no difference between the Syk score in the lamina propria and serum IgE (data not shown). Nine NACS samples which has IgE data were included.

4. Discussion

In this study, we demonstrated that Syk expression in nasal polyps from AR patients was significantly higher than that those from NACS patients. There was no difference in the Syk expression between nasal mucosa from AR and that from NACS. The Syk-positive cells are mainly eosinophils in the nasal polyp. Syk was stained in the cytoplasm of eosinophils. The mean percent of Syk positive cells in nasal polyps were associated with serum total IgE.

Eosinophils are well known to be induced and activated by several cytokines and chemokines. In allergic disease, the up-regulation of Interleukin-5 (IL-5), granulocyte/macrophage colony-stimulating factor (GM-CSF), eotaxin and RANTES cause blood and tissue eosinophilia. Patients with allergic nasal polyposis had significantly higher tissue densities of IL-4, IL-5, GM-CSF and IL-3 compared with those with non-allergic nasal polyposis [21,22]. Fan et al. also reported that EG2 and IL-5 positive cells were abundant in the submucosa of patients with allergic sinusitis, especially in the superficial layer. About half of the IL-5 producing cells were eosinophils and apoptotic eosinophils were less numerous in the superficial layer [23]. Human eosinophils have IL-3/IL-5/GM-CSF receptor on