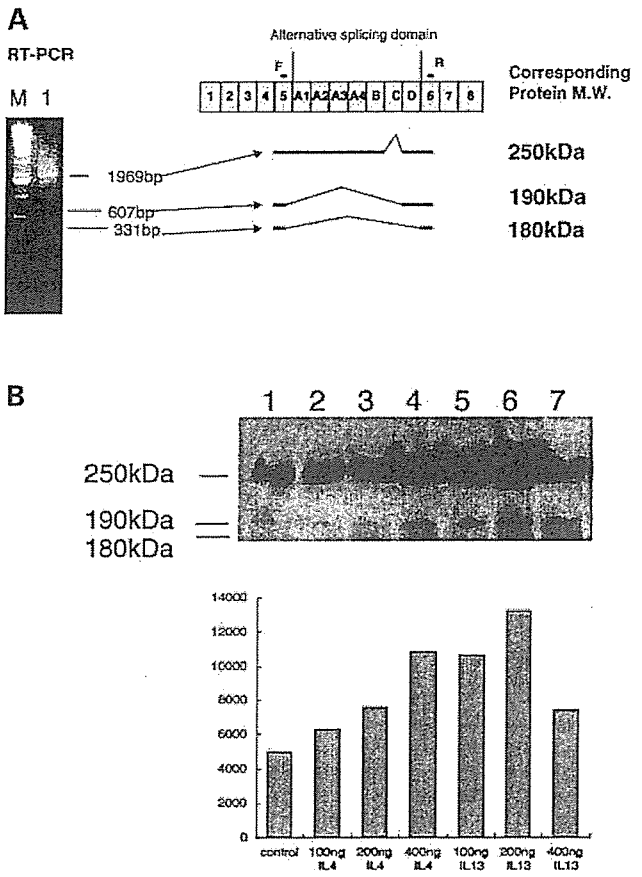

Figure 5. RT–PCR and western blot analysis for TNC variants in NHLF with Th2 cytokine stimulation. (A) TNC mRNA expression in NHLF was examined with RT–PCR. Subconfluent NHLF were stimulated with 100 ng/ml IL-13 for 72 h. After mRNA extraction and cDNA synthesis, PCR was performed with a pair of primers designed to differentiate alternatively spliced mRNA of TNC. The PCR products were electrophoresed on 1% agarose gel. M: 1 kb DNA marker and Lane 1: NHLF (left side). The structure of the FN-III domain in the human TNC gene is shown (center). F: forward primer and R: reverse primer. Corresponding protein molecular weights (M.W.) are indicated to the right. (B) Western blot analysis of NHLF culture samples stimulated with IL-4 or with IL-13 for 72 h. The samples were electrophoresed in 4–20% Tris–glycine gels and electrotransferred in PVDF membrane. Immunoblotting was performed with the rat anti-TNC monoclonal antibody. The relative intensity of TNC protein was quantified with NIH Image and is shown at the bottom. Lane 1 represents NHLF without stimulation. Lanes 2–4 represent NHLF stimulated with IL-4 at the concentrations of 100, 200 and 400 ng/ml, respectively. Lanes 5–7 represent NHLF stimulated with IL-13 at the concentrations of 100, 200 and 400 ng/ml, respectively.

Biosystems). We also utilized the SNP information from the database of SNPs by Japanese Science and Technology Agency database (JSNP). Intragenic pairwise LD in the TNC locus was examined by measuring r^2 among 22 SNPs. The pairwise LD and haplotype were evaluated using the SNPAlyze 3.1 software (Dynacom Co. Ltd, Chiba, Japan). Position SNPs were numbered according to their position relative to the published genomic sequence containing the TNC region (GenBank accession no. AL162425), and position 1 is the adenine of the first methionine of TNC. The panel of 10 SNPs was genotyped with the multiplex PCR-Invader assay or Taqman genotyping system as described previously (27). To investigate the pattern of LD in and around the TNC locus, pairwise LD coefficients were calculated and expressed as $|D'|$ or r. We evaluated the LD extension of the TNC genomic region with 48 SNPs registered in JSNP by genotyping 1041 general Japanese subjects.

Statistical analysis

Allele frequencies in bronchial asthma and controls were compared by the contingency χ^2 test. A P-value of less than 0.01, after Bonferroni adjustment in case of multiple comparisons, was considered to be statistically significant. The OR and 95% CI were also calculated. Haplotype frequencies were estimated by the expectation-maximization algorithm.

TNC immunohistochemistry

TNC immunohistochemistry was performed essentially as previously described (24). Fresh human lung tissues were obtained and embedded in paraffin from patients undergoing surgery; informed consent was obtained. Asthmatic lung specimen was obtained from autopsied lung. The sections were deparaffined and endogenous peroxidase activity was quenched with 0.3% H_2O_2 in methanol for 20 min. Non-specific staining was blocked with blocking buffer [10% normal goat serum and 1% bovine serum albumin in phosphate-buffered saline (PBS)] for 30 min. The rat anti-human TNC antibody (10 μg/ml) was applied and reacted overnight at 4°C. After PBS washing, slides were incubated with HRP-conjugated anti-rat IgG antibody for 30 min. The slides were developed with DAB (Dojindo, Kumamoto, Japan) in Tris-buffered saline with 0.05% H_2O_2.

Computer modeling of TNC Fn-III-D protein structure

To examine the effect of amino acid substitution at position 1677 in the Fn-III-D domain, protein structural modeling was performed using MOE software (Chemical Computing Group Inc., Montreal, Canada). The coordinates of the 2.25 Å crystal structure of the chicken TNC Fn-III domain (PDB accession no. P24821) were used as a template for homology modeling of the human TNC Fn-III-D domain. The two structures were further minimized with AMBER 94 using MOE software. Both Leu1677 Ile variants of the TNC Fn-III-D domain were built up using the same program.

RT–PCR and western blotting analysis for TNC variants detection

Subconfluent NHLF were stimulated with 100 ng/ml IL-13 for 72 h and mRNA was isolated using a QuickPrep micro mRNA purification kit (Amersham, Uppsala, Sweden). cDNA was made with the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) using oligo$(dT)_{20}$ primer. RT–PCR was carried out for 5 min at 95°C for initial denaturing, followed by 35 cycles of 95°C for 60 s, 52°C for 60 s, and 72°C for 120 s, in the GeneAmp PCR System 9700 (Applied Biosystems). The primer TNC-3089: ACCGCTACCGCCT

CAATTACA and TNC-5331: GGTTCCGTCCACAGT TACCA were set to distinguish mRNA variants due to alternative splicing (13). The PCR products were electrophoresed in 1% agarose gel and distinct bands were excised. DNA was extracted from the excised bands with a DNA Gel Extraction Kit (Millipore, Tokyo, Japan) and subcloned into pCR II-TOPO cloning vector (Invitrogen). The subcloned inserts were read by sequencing. For western blotting, subconfluent NHLF were stimulated either with IL-4 or with IL-13 for 72 h at the concentration indicated in Fig. 5. The NHLF were solublized with SDS sample buffer (50 mM Tris–HCl pH6.8, 2% SDS, 20% glycerol, 0.4% bromophenol blue, 50 mM DTT). SDS–PAGE and subsequent immunoblotting were performed as previously described (21).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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Conflict of Interest statement. None declared.

REFERENCES

1. Callerame, M.L., Condemi, J.J., Bohrod, M.G. and Vaughan, J.H. (1971) Immunologic reactions of bronchial tissues in asthma. *N. Engl. J. Med.*, **284**, 459–464.
2. Carroll, N., Elliot, J., Morton, A. and James, A. (1993) The structure of large and small airways in nonfatal and fatal asthma. *Am. Rev. Respir. Dis.*, **147**, 405–410.
3. Cohn, L., Elias, J.A. and Chupp, G.L. (2004) Asthma: mechanisms of disease persistence and progression. *Annu. Rev. Immunol.*, **22**, 789–815.
4. Heinzmann, A., Mao, X.Q., Akaiwa, M., Kreomer, R.T., Gao, P.S., Ohshima, K., Umeshita, R., Abe, Y., Braun, S., Yamashita, T. *et al.* (2000) Genetic variants of IL-13 signalling and human asthma and atopy. *Hum. Mol. Genet.*, **9**, 549–559.
5. Van Eerdewegh, P., Little, R.D., Dupuis, J., Del Mastro, R.G., Falls, K., Simon, J., Torrey, D., Pandit, S., McKenny, J., Braunschweiger, K. *et al.* (2002) Association of the *ADAM33* gene with asthma and bronchial hyperresponsiveness. *Nature*, **418**, 426–430.
6. Mitsuyasu, H., Izuhara, K., Mao, X.Q., Gao, P.S., Arinobu, Y., Enomoto, T., Kawai, M., Sasaki, S., Dake, Y., Hamasaki, N. *et al.* (1998) Ile50Val variant of IL4R alpha upregulates IgE synthesis and associates with atopic asthma. *Nat. Genet.*, **19**, 119–120.
7. Flood-Page, P., Menzies-Gow, A., Phipps, S., Ying, S., Wangoo, A., Ludwig, M.S., Barnes, N., Robinson, D. and Kay, A.B. (2003) Anti-IL-5 treatment reduces deposition of ECM proteins in the bronchial subepithelial basement membrane of mild atopic asthmatics. *J. Clin. Invest.*, **112**, 1029–1036.
8. Laitinen, A., Altraja, A., Kampe, M., Linden, M., Virtanen, I. and Laitinen, L.A. (1997) Tenascin is increased in airway basement membrane of asthmatics and decreased by an inhaled steroid. *Am. J. Respir. Crit. Care Med.*, **156**, 951–958.
9. Yuyama, N., Davies, D.E., Akaiwa, M., Matsui, K., Hamasaki, Y., Suminami, Y., Yoshida, N.L., Maeda, M., Pandit, A., Lordan, J.L. *et al.* (2002) Analysis of novel disease-related genes in bronchial asthma. *Cytokine*, **19**, 287–296.
10. Lee, J.H., Kaminski, N., Dolganov, G., Grunig, G., Koth, L., Solomon, C., Erle, D.J. and Sheppard, D. (2001) Interleukin-13 induces dramatically different transcriptional programs in three human airway cell types. *Am. J. Respir. Cell Mol. Biol.*, **25**, 474–485.
11. Oberhauser, A.F., Marszalek, P.E., Erickson, H.P. and Fernandez, J.M. (1998) The molecular elasticity of the extracellular matrix protein tenascin. *Nature*, **393**, 181–185.
12. Chiquet-Ehrismann, R., Tannheimer, M., Koch, M., Brunner, A., Spring, J., Martin, D., Baumgartner, S. and Chiquet, M. (1994) Tenascin-C expression by fibroblasts is elevated in stressed collagen gels. *J. Cell Biol.*, **127**, 2093–2101.
13. Siri, A., Carnemolla, B., Saginati, M., Leprini, A., Casari, G., Baralle, F. and Zardi, L. (1991) Human tenascin: primary structure, pre-mRNA splicing patterns and localization of the epitopes recognized by two monoclonal antibodies. *Nucleic Acid. Res.*, **19**, 525–531.
14. NIH (1995) Global Initiative for Asthma. *NHLBI Global Strategy for Asthma Management and Prevention NHLBI/WHO Workshop.* NIH publication, Bethesda, MD.
15. Wjst, M., Fischer, G., Immervoll, T., Jung, M., Saar, K., Rueschendorf, F., Reis, A., Ulbrecht, M., Gomolka, M., Weiss, E.H. *et al.* (1999) A genome-wide search for linkage to asthma. German Asthma Genetics Group. *Genomics*, **58**, 1–8.
16. Kaarteenaho-Wiik, R., Kinnula, V., Herva, R., Paakko, P., Pollanen, R. and Soini, Y. (2001) Distribution and mRNA expression of tenascin-C in developing human lung. *Am. J. Respir. Cell Mol. Biol.*, **25**, 341–346.
17. Kaarteenaho-Wiik, R., Kinnula, V.L., Herva, R., Soini, Y., Pollanen, R. and Paakko, P. (2002) Tenascin-C is highly expressed in respiratory distress syndrome and bronchopulmonary dysplasia. *J. Histochem. Cytochem.*, **50**, 423–431.
18. Roth-Kleiner, M., Hirsch, E. and Schittny, J.C. (2004) Fetal lungs of tenascin-C-deficient mice grow well, but branch poorly in organ culture. *Am. J. Respir. Cell Mol. Biol.*, **30**, 360–366.
19. Young, S.L., Chang, L.Y. and Erickson, H.P. (1994) Tenascin-C in rat lung: distribution, ontogeny and role in branching morphogenesis. *Dev. Biol.*, **161**, 615–625.
20. Matlin, A.J., Clark, F. and Smith, C.W. (2005) Understanding alternative splicing: towards a cellular code. *Nat. Rev. Mol. Cell Biol.*, **6**, 386–398.
21. Matsuda, A., Yoshiki, A., Tagawa, Y., Matsuda, H. and Kusakabe, M. (1999) Corneal wound healing in tenascin knockout mouse. *Invest. Ophthalmol. Vis. Sci.*, **40**, 1071–1080.
22. Koyama, Y., Kusubata, M., Yoshiki, A., Hiraiwa, N., Ohashi, T., Irie, S. and Kusakabe, M. (1998) Effect of tenascin-C deficiency on chemically induced dermatitis in the mouse. *J. Invest. Dermatol.*, **111**, 930–935.
23. Nakao, N., Hiraiwa, N., Yoshiki, A., Ike, F. and Kusakabe, M. (1998) Tenascin-C promotes healing of habu-snake venom-induced glomerulonephritis: studies in knockout congenic mice and in culture. *Am. J. Pathol.*, **152**, 1237–1245.
24. Matsuda, A., Tagawa, Y. and Matsuda, H. (1999) TGF-beta2, tenascin, and integrin beta1 expression in superior limbic keratoconjunctivitis. *Jpn. J. Ophthalmol.*, **43**, 251–256.
25. American Thoracic Society. (1962) Chronic bronchitis, asthma and pulmonary emphysema: a statement by the Committee on Diagnostic Standards for Nontuberculous Respiratory Diseases. *Am. Rev. Respir. Dis.*, **85**, 762–768.
26. Fujita, K., Kasayama, S., Hashimoto, J., Nagasaka, Y., Nakano, N., Morimoto, Y., Barnes, P.J. and Miyatake, A. (2001) Inhaled corticosteroids reduce bone mineral density in early postmenopausal but not premenopausal asthmatic women. *J. Bone Miner. Res.*, **16**, 782–787.
27. Ohnishi, Y., Tanaka, T., Ozaki, K., Yamada, R., Suzuki, H. and Nakamura, Y. (2001) A high-throughput SNP typing system for genome-wide association studies. *J. Hum. Genet.*, **46**, 471–477.

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Association of Transforming Growth Factor-β1 Single Nucleotide Polymorphism C-509T with Allergy and Immunological Activities

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Key Words
Immunological activities · Single nucleotide polymorphisms · T-cell proliferation · TGFβ1

Abstract

Background: A single nucleotide polymorphism (SNP) C-509T within the tumor growth factor β1 (TGFβ1) gene has been associated with atopic asthma and asthma severity. To further understand the mechanisms involved, the association of C-509T with allergy, T-lymphocyte proliferation and plasma TGFβ1 concentration has been explored in a case-control study with allergic and nonallergic subjects. ***Methods:*** The recruited subjects including allergic (n = 38) and nonallergic (n = 25) participants have been genotyped for C-509T using allele discrimination assay. Association of C-509T with allergy status was examined using logistic regression analysis in both dominant and recessive models. Association of C-509T with T-cell proliferation in control and antigen-stimulated peripheral blood mononuclear cells (PBMCs), plasma TGFβ1 and total IgE level were tested by multiple regression analysis. ***Results:*** Individuals with homozygous mutant TT genotype showed a higher risk of allergy (TT: odds ratio = 5.099, 95% confidence limit: 1.355–19.190, p = 0.016) after covariates were adjusted. A trend to increased plasma TGFβ1 in subjects with T allele has been discovered. In the meantime, the T allele is associated with lower T cell proliferation in controls and maximum response to above antigens. A low T-cell proliferation is correlated with higher plasma TGFβ1 concentration ($p < 0.01$). The in vitro studies confirmed the suppressing effect of TGFβ1 on T-cell proliferation at physiological range. A significant inhibitory effect on IL-4 production was also observed. ***Conclusions:*** A C to T base change in TGFβ1 SNP C-509T has been associated with a higher risk of allergy. The mechanisms are not clear. Elevated TGFβ1 levels associated with the C-509T polymorphism might suppress immune activation as well as Th2 cytokine production.

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Introduction

Allergic diseases such as asthma, allergic rhinitis and atopic dermatitis have been considered as complex polygenic disorders resulting from a combination of genetic and environmental factors. In past years, a lot of efforts have been pursued to look for possible genetic components contributing to allergies and asthma [1–4]. Studies from our group and others have indicated that tumor

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growth factor β1 (TGFβ1) is a promising candidate gene whose genetic polymorphisms may play a key role in allergic diseases [5–10]. Among reported genetic polymorphisms in the TGFβ gene, a C to T base exchange at –509 position in the promoter region was associated with elevated levels of total IgE in allergic asthma patients in our previous study [8]. Two subsequent independent case-control studies in relatively large populations reported the association of variant T allele with asthma, and even with asthma severity [5, 6]. The exact role and possible mechanisms involved are still not clear.

TGFβ1 is a 25-kDa disulfide-linked homodimeric multifunctional cytokine. With respect to its diverse activities in normal cell growth and development, TGFβ1 might be the most important growth factor in immunomodulatory effects because knockout mice died of massive inflammatory lesions [7]. Both anti-inflammatory and inflammatory properties have been reported [8]. In the present case-control study including allergic and non-allergic subjects, we have been able to further study the significances of the TGFβ1 C-509T genetic polymorphism in allergic diseases, its association with TGFβ1 production, immune modulation activity reflected from antigen-stimulated T-cell proliferation in peripheral blood mononuclear cells (PBMCs), and even regulatory effects on cytokine production.

Patients and Methods

Subjects

Adult volunteers either with a history of atopy or allergic diseases, e.g. allergic rhinitis, atopic dermatitis, and asthma, or no allergy (healthy individuals) have been recruited. Atopy or allergy status was determined based on identified allergen through positive skin test or allergen-specific IgE test, and allergic symptoms. To avoid possible confounding effects for T-cell proliferation to antigen, patients with other diseases especially an immune deficiency, allergic patients with a history of immunotherapy, and patients who have taken steroids or other immunosuppressive agents at least 3 days before blood drawing met the exclusion criteria. All subjects signed informed consent with National Jewish Institutional Review Board approval before being enrolled and donating blood.

Genotyping

Peripheral venous blood was obtained from each subject. Genomic DNA was extracted from whole blood using the QIAamp blood kit (QIAGEN, Santa Clarita, Calif., USA). Briefly, blood cells were first lysed, and supernatant containing genomic DNA was mixed with ethanol and applied to QIAamp spin column. After washing several times, purified genomic DNA attached in column was eluted finally in TE buffer.

The allelic discrimination assay using the ABI PRISM 7700 Sequence Detection System (AB Applied Biosystems, Foster City, Calif., USA) has been developed for genotyping of TGFβ1 C-509T (HUgn0007040). Primers used for amplification of DNA fragments are: forward primer at position –572~–551 (-CATGGGAGGT-GCTCAGTAAAGG-), reverse primer at position –437~–454 (-GGTAGGAGAAGAGGGTCTGTCAAC-). Probe 1 containing allele C at position –519~–494 was linked with reporter dye FAM (6-carboxyfluorescein) at the 5′ end and black hole quencher (Biosearch Technologies) at the 3′ end: -CCCTTCCATCCCT-CAGGTGTCCTGTT-. Probe 2 containing allele T instead of C was labeled with reporter dye 6-JOE (6-carboxy-4′,5′-dichloro-2′,7′-dimethoxyfluorescein). Each reaction contains 25 μl of Taqman Universal Master Mix (AB Applied Biosystems), 900 n*M* of forward and reverse primers in concentration, 100 n*M* of probe 1 and probe 2 in concentration, and 200 ng of genomic DNA in 50 μl total volume. Thermal cycling conditions for allelic discrimination assay are: 2 min at 50°C, 10 min at 95°C for AmpliTaq Gold Activation, and 40 cycles of 15 s at 95°C for denaturing and 1 min at 60°C for annealing/extension. Rn for a given reaction tube, defined as the ratio of the emission intensity of the reporter dye over the emission intensity of the passive reference, was obtained. The relative Rn value of FAR to JOE reporter dye for each reaction was utilized to separate CC (1.1–1.43), CT (0.74–1.1) and TT (0.30–0.74) after adjustment with non-template control based on the 95% confidence limits in each group. Duplicate experiments have been performed confirming reproducibility. Three distinct groups for CC, CT and TT have been recognized based on our setup thresholds.

Cell Culture and Cell Proliferation

PBMCs were isolated from human heparinized venous blood by density gradient centrifugation on Ficoll-Paque (Amersham, Uppsala, Sweden), and washed three times in HBSS (Gibco, Grand Island, N.Y., USA). Purified PBMCs were suspended in complete RPMI 1640 medium (Gibco), which was supplemented with 5% heat-inactivated fetal calf serum (Gemini Bio-Products, Calabasas, Calif., USA), 200 U/ml penicillin, 200 μg/ml streptomycin, 50 μg/ml gentamicin, 4 m*M* *L*-glutamine (Gibco), 10 m*M* HEPES (Sigma), 1 m*M* sodium pyruvate (Gibco), 0.1 m*M* nonessential amino acid (Gibco), and 50 μ*M* 2-mercaptoethanol.

To assess lymphocyte proliferation response, PBMCs were cultured at a concentration of 10^5 cells/well in 96-well flat-bottom plates in a volume of 200 μl. PBMCs were incubated with medium control; tetanus toxoid (Aventis Pasteur, Swiftwater, Pa., USA) at concentrations of 0.02, 0.07, and 0.2 lac-forming units (LFU)/ml; timothy (pollen) *Phleum pratense* allergic extract (Bayer, Elkhart, Ind., USA) at concentrations of 4, 40, and 400 PNU/ml, and standardized cat hair allergic extract (Fel d1; ALK, Horsholm, Denmark) of 1, 10, and 100 BAU/ml. On day 6, 1.0 μCi/well [^{3}H] thymidine (Amersham, Arlington Heights, Ill., USA) was added to 100 μl of cell culture and incubated for 6 additional hours. Cells were finally harvested onto filter paper disks via a multiwell harvester and radioactivity (counts/min) counted with a 1450 Microbeta Plus Liquid Scintillation Counter (Wallace, Orange, Calif., USA). All samples were run in triplicate.

To assess the effect of TGFβ1 on cell proliferation, PBMCs were also cultured at a concentration of 10^5 cells/well in 96-well flat-bottom plates in a volume of 200 μl for 6 days. Human recombinant TGFβ1 (R&D Systems, Minneapolis, Minn., USA) was added as indicated in the Results. Radioactivity was counted as described above.

To assess the effect of TGFβ1 on cytokine production, PBMC were cultured at a concentration of 10^6 cells/well and treated as described in the Results. Supernatant was collected at 48 h, and IL-2, IFNγ, IL-4 were measured using ELISA assays.

Plasma TGFβ1 Measurement

Plasma was first isolated from heparinized venous blood by density gradient centrifugation on Ficoll-Paque and further centrifuged at 3,000 *g* for 10 min to eliminate platelets, and then frozen at –70°C. Plasma TGFβ1 was measured using the TGFβ1 ELISA Kit (Biosource, Nivelles, Belgium). Based on the manufacturer's instruction, latent TGFβ1 was first extracted from platelet-depleted plasma, and incubated with biotinylated anti-TGFβ1 in TGFβ1-antibody-coated wells for 3 h as well as TGFβ1 standard and diluent control. Streptavidin-HRP working solution was added for 30 min after thorough washing. Stabilized chromogen solution was finally added to each well for 30 min and terminated by stopping solution before absorbance (optical density, OD) was detected at a wavelength of 450 nm. The concentration of plasma TGFβ1 was calculated after deriving from a standard curve. The minimum detectable dose of TGFβ1 is 15.6 pg/ml. Duplicate experiments have been performed.

Plasma Total IgE Measurement

Plasma total IgE was measured using the ELISA kit from ALerCHEK (Portland, Maine, USA). According to the manufacturer's instructions, 20 μl of standard IgE and plasma sample were diluted with 80 μl of specimen diluent and incubated in anti-human IgE-coated microwells for 60 min at room temperature. After thorough washing with washing buffer, 100 μl of HRP-conjugated goat anti-human IgE was added for another 60 min. Finally, 100 μl of TMB/peroxide substrate were incubated for 30 min after another thorough washing and terminated by 0.5 *N* sulfuric acid before OD at 450 nm was determined. Total IgE concentration (IU/ml) in plasma was derived from the constructed standard curve. Duplicate experiments have been performed.

Cytokine Assays

IL-2, IFNγ and IL-4 production in cell culture supernatants was measured using ELISA kits (Biosource) with a sensitivity of <5 pg/ml, 0.03 IU/ml, and 2 pg/ml, respectively. According to the instructions, standard, control and samples from supernatant were incubated in specific-antibody-coated microwells together with antibody conjugate for 2 h at room temperature on a horizontal shaker set. After thorough washing, 200 μl of freshly prepared chromogenic solution were pipetted into each well and incubated for 15 min at room temperature on a horizontal shaker set before 50 μl of stop solution were added. The OD value at 450 nm was read, and plasma concentrations of cytokines were calculated based on standard curves finally.

Statistical Methods

Statistical analyses were performed using SAS software version 9.1.3. Balance of categorical variables regarding gender, race, ethnicity, genotype and allele frequency between allergy and non-allergy groups was tested using the χ^2 test or Fisher's exact test in PROC FREQ. The Hardy-Weinberg equilibrium for TGFβ1 C-509T allele frequency was calculated according to Thompson et al. [9]. Logistic regression was utilized to find the association between the TGFβ1 C-509T genotype and allergy status, while gender (male or female), race (White, Asian or Black) and TGFβ1 C-509T genotype (CC and CT, or TT only) has been considered in model building. Backward selection with slentry = 0.2, slstay = 0.2 options was used to remove low-risk factors. To adjust for possible confounding influences in the association of the C-509T genotype with numerical variables such as T-cell proliferation, stepwise-wise selection with slentry = 0.2 and slstay = 0.2 has been run with race, gender, allergy status, and TGFβ1 C-509T genotype entered in multiple regression models. Regression diagnostics have been performed to make sure that no violation of assumption occurs. Pearson's correlation coefficient has been used to estimate correlations between two numerical variables. Nonparametric approaches (the Kruskal-Wallis test and Wilcoxon's two-sample test) have been applied for multiple comparison among groups. p values less than 0.05 are considered as statistically significant.

Results

We recruited 63 volunteers including 38 with a history of allergic or atopic diseases and 25 normal subjects. All of them are nonsmokers. The properties of the recruited population are summarized in table 1. There is no significant difference between allergy and non-allergy groups regarding the composition of gender, race, ethnicity (χ^2 test or Fisher's exact test, $p > 0.05$) and age (Wilcoxon's rank sum test, $p > 0.05$). The allele frequency of TGFβ1 C-509T is consistent with the Hardy-Weinberg equilibrium ($\chi^2 = 3.93$, $p > 0.1$). Except for medium and phytohemagglutinin (PHA) control, T-cell proliferation stimulated by tetanus toxoid and timothy allergy is significantly higher in the allergy group than in the non-allergy group. There is a trend towards an increase in T-cell proliferation for cat hair allergen in the allergy group although it is not statistically significant ($p = 0.0710$).

Association of the TGFβ1 C-509T Genotype with Allergy

The association of the TGFβ1 C-509T genotype with allergy was examined using logistic regression. The possible covariates such as gender (male or female) and race (White, Asian or Black) have been considered and were removed according to the selection criterion (see Statistical Analysis). In the recessive model, the allergy status was associated with the TT genotype (odds ratio = 5.099, 95% confidence limits 1.355–19.190, $p = 0.016$). However, there is no significant association between allergy status and CC in the dominant model (odds ratio = 0.823, 95% confidence limits 0.26–2.383, $p = 0.7199$). This suggests that the TT variant of the TGFβ1 gene at promoter –509 is associated with increased risk of allergy in humans.

Table 1. Subject characteristics (mean and SD)

	Allergy	No allergy	Total	p value
Subjects	38	25	63	
Age, years	36 (10)	38 (12)	36 (11)	0.5737
Sex, %				
Female	58	52	56	0.6450
Race, %				
White	79	68	75	
Asian	16	32	22	
Black	5	0	3	0.2725
Ethnicity, %				
Non-hispanic	89	100	94	
Hispanic	11	0	6	0.0937
TGFβ1 C-509T genotype, %				
CC	37	44	40	
CT	53	20	40	
TT	11	36	20	0.0158
TGFβ1 C-509T allele frequency, %				
C	63	54	60	
T	37	46	40	0.9114
Timothy allergy, %				
Yes	84	0	51	<0.0001
Cat allergy, %				
Yes	58	0	35	<0.0001
CPM for control	8.49 (1.64)	8.12 (1.34)	8.34 (1.52)	0.2243
CPM for tetanus toxoid	9.70 (1.13)	9.04 (1.12)	9.44 (1.16)	0.0216
CPM for cat hair allergen	9.19 (1.36)	8.59 (1.13)	8.95 (1.30)	0.0710
CPM for timothy allergen	9.36 (1.15)	8.79 (1.08)	9.13 (1.15)	0.0409
CPM for PHA	11.45 (0.66)	11.45 (0.51)	11.45 (0.60)	0.8088
Total IgE	1.58 (0.75)	0.84 (0.72)	1.32 (0.82)	0.0009
Plasma TGFβ1, ng/ml	3.10 (0.47)	3.16 (0.41)	3.12 (0.45)	0.6014

Racial and ethnic classification is according to the standard used in Census 2000. For age and other numerical data, means (SD) are shown. Values for CPM and total plasma IgE are natural logarithmic transformed. Wilcoxon's rank sum score test was used for comparisons between allergy and non-allergy groups.

Association of TGFβ1 C-509T with Plasma TGFβ1 Level

To understand the possible mechanisms associated with the above finding, the association of C-509T with plasma TGFβ1 levels has also been estimated using multiple regression. The results indicated that there is a trend to higher plasma TGFβ1 concentration in the presence of T at the C-509T allele (CC: 3,008.34 ± 369.96, CT: 3,143.76 ± 529.17, and TT: 3,312.66 ± 386.55 pg/ml). However, statistically significant differences could not be shown in either the dominant model (CC only: p = 0.1198) or the recessive model (TT only: p = 0.1346) while possible covariates such as allergy, gender, age and race have been adjusted for. The hypothesis that a higher concentration of plasma TGFβ1 in the TT genotype accounts for the association of C-509T with allergy may need to be verified in larger studies in the future.

Association of the TGFβ1 C-509T Genotype with T-Cell Proliferation in Human PBMCs

With respect to the importance of TGFβ1 in the immunomodulation of allergen-induced cell proliferation, the association of the TGFβ1 C-509T genotype with antigen-stimulated cell proliferation in human PBMCs has also been examined using multiple regression analysis. Based on selection rules, the adjusted variables are listed in table 2 as indicated. Outcomes including unstimulated cell proliferation (medium control) and cell proliferation induced by maximum tetanus toxin, cat hair allergen and timothy allergen extracts showed a negative relationship

Table 2. Association of the TGFβ1 C-509T genotype with T-cell proliferation and IgE (n = 63)

Outcomes (ln value)	Adjusted variables	Regression coefficients for TT or CC (mean ± SE)	t value	p value
TT (recessive)				
CPM for medium control	White	−1.26 ± 0.48	−2.64	0.0127
CPM for tetanus toxoid	Black	−1.04 ± 0.34	−3.08	0.0031
CPM for cat hair allergen	cat allergy, White	−1.02 ± 0.41	−2.50	0.0152
CPM for timothy allergen	grass allergy, White	−0.93 ± 0.36	−2.55	0.0134
CPM for PHA	age, White	−0.07 ± 0.192	−0.37	0.7132
Plasma IgE	allergy	0.003 ± 0.26	0.01	0.9902
CC (dominant)				
CPM for medium control	age, White, Asian	1.20 ± 0.37	3.25	0.0019
CPM for tetanus toxoid	age, allergy	0.70 ± 0.28	2.48	0.0159
CPM for cat hair allergen	White, cat allergy	0.67 ± 0.32	2.07	0.0429
CPM for timothy allergen	White, grass allergy	0.84 ± 0.29	2.95	0.0047
CPM for PHA	age, Asian	0.03 ± 0.16	0.19	0.8464
Plasma IgE	allergy	0.07 ± 0.20	0.34	0.7376

with TT ($p < 0.05$) in the recessive model except for PHA. Conversely, the T-cell proliferation had a positive relationship with CC in the dominant model ($p < 0.05$) except for PHA. These results indicate that the C-T mutation is associated with reduced T-cell proliferation in general. We have used the stimulation index (ratio of antigen-stimulated cell proliferation over control) as response variable for tetanus toxin, cat allergen and timothy allergen. Neither the TT nor the CC genotype was correlated with antigen-specific cell proliferation (data not shown), suggesting that suppression might not be antigen specific.

The analysis for the association of the TGFβ1 C-509T genotype with plasma total IgE indicates that IgE is apparently not associated with the TGF C-509T genotype ($p = 0.9902$ for TT and $p = 0.7376$ for CC). The C-509T genotype does not exhibit an allergy-status-dependent difference ($p = 0.5676 > 0.05$ for TT and $p = 0.6718 > 0.05$ for CC) in IgE level.

Effect of TGFβ1 on T-Cell Proliferation

We also tested whether higher concentration of plasma TGFβ1 is correlated with lower T-cell proliferation capacity (fig. 1). From the graphs, negative associations of the plasma TGFβ1 concentration with natural log-transformed value of control, tetanus toxoid, cat allergen, timothy allergen, and even PHA-stimulated cell proliferation were found. Total IgE seems not to be associated with plasma TGFβ1 amounts. After adjusting for possible covariates such as gender, race and age in multiple regression models, the linear correlation still exists (table 3). These results support our hypothesis that human subjects with the T allele in the TGFβ1 C-509T genetic polymorphism suppressed immunological activity resulting probably from higher TGFβ1 production.

Table 3. Regression analysis of T-cell proliferation and IgE with plasma TGFβ1 concentration adjusted for gender, age, and race (n = 63)

Outcomes (ln value)	Regression coefficients for TGFβ1 (mean ± SE), $\times 10^{-3}$	t value	p value
CPM for medium control	−1.573 ± 0.4251	−3.70	0.0005
CPM for tetanus toxoid	−0.808 ± 0.3354	−2.41	0.0196
CPM for cat hair allergen	−1.239 ± 0.3711	−3.34	0.0016
CPM for timothy allergen	−0.970 ± 0.3317	−2.92	0.0051
CPM for PHA	−0.356 ± 0.1924	−1.85	0.0705
Plasma IgE	−0.138 ± 0.2291	−0.6	0.5485

Furthermore, recombinant human TGFβ1 was added at three different concentrations (32 and 3.2 ng/ml and 32 pg/ml) simultaneously in in vitro studies while PBMCs were cultured in the presence of tetanus toxoid (0.02 LFU/ml) as a stimulator. TGFβ1 significantly inhibited cell proliferation at 32 ng/ml ($p = 0.012$) with a dose-response trend (fig. 2) consistent with a suppressing effect of TGFβ1.

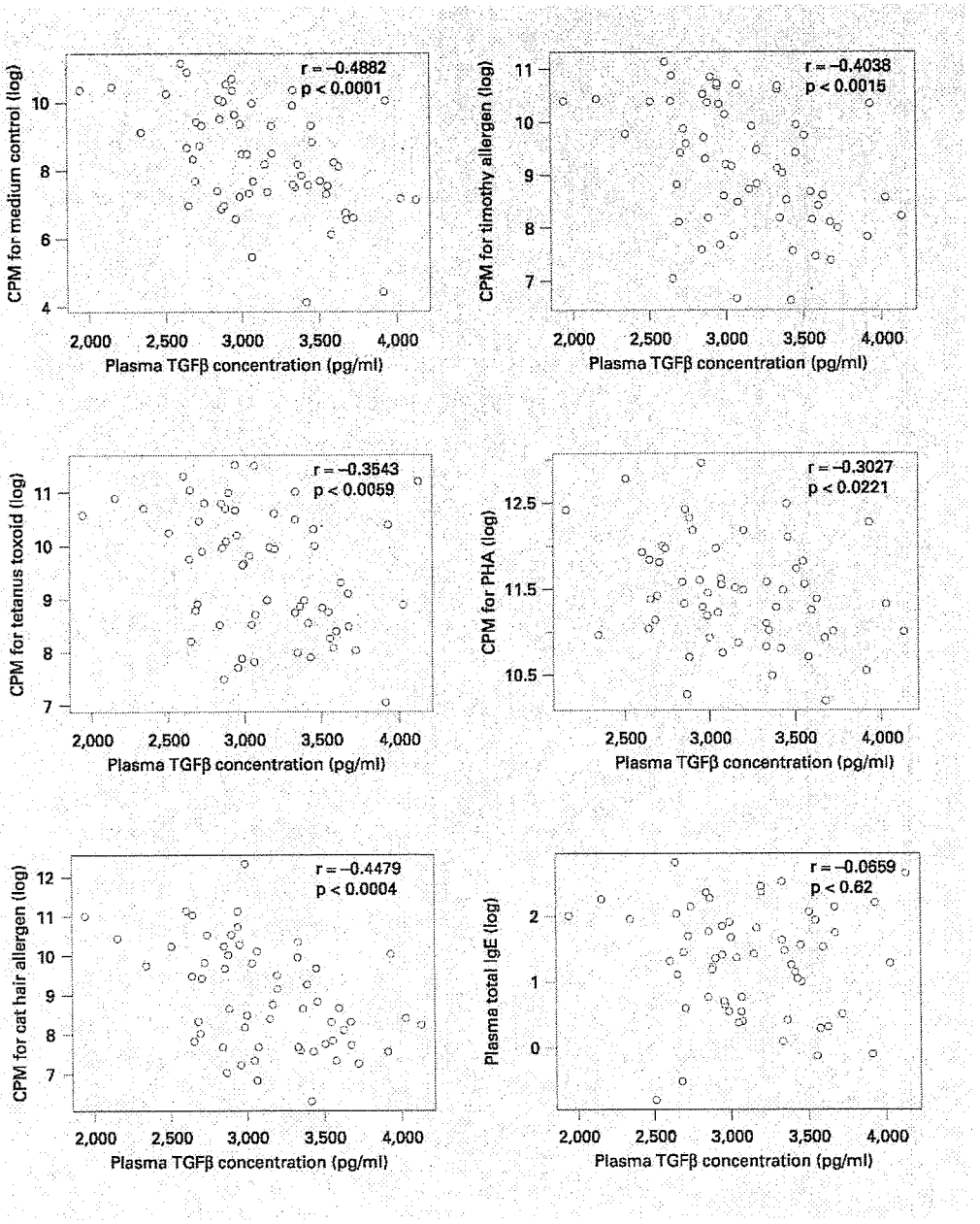

Fig. 1. Scatter plot of plasma TGFβ1 with plasma total IgE and T-cell proliferation in response to antigens in 63 subjects. Counts per minute (CPM) and total IgE are the natural log-transformed value of maximum T-cell proliferation in medium control, tetanus toxoid, cat allergen, timothy allergens, PHA-stimulated PBMCs and plasma IgE level. r = Correlation coefficient.

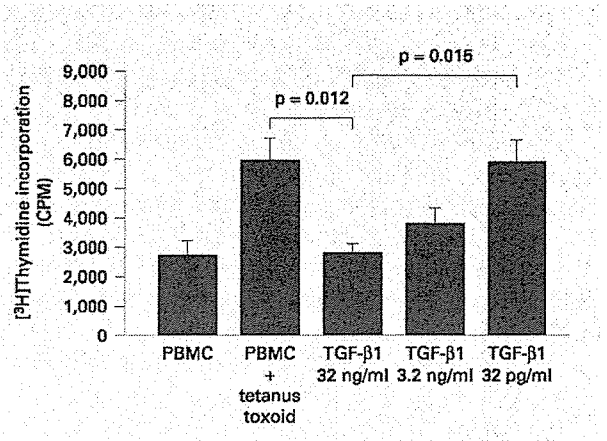

Fig. 2. Inhibition of T-cell proliferation by TGFβ1. [^{3}H]thymidine uptake (counts/min. CPM) of T-cells stimulated with 0.02 LFU/ml tetanus toxoid in the presence of TGFβ1 for 6 days (n = 8).

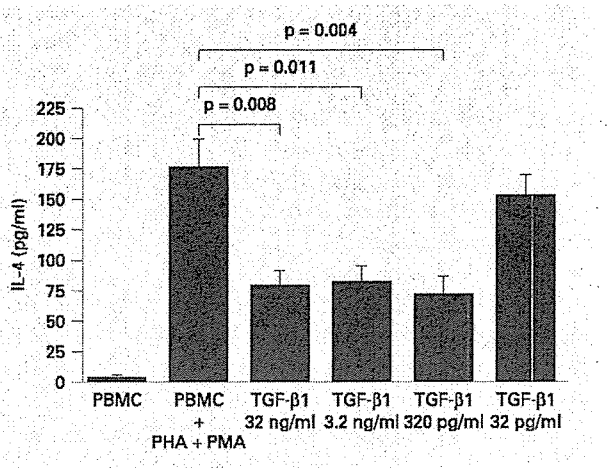

Fig. 3. Inhibition of IL-4 synthesis by TGFβ1. PBMCs were stimulated with PHA 10 μg/ml and 10 ng/ml phorbol 12-myristate 13-acetate (PMA) in the presence of TGFβ1 (n = 5).

Inhibiting Effect of TGFβ1 on Cytokine Production

Cytokines have been considered to be very important in regulating T-cell proliferation. The inhibitory effect of TGFβ1 on cytokine production was further studied in vitro. PBMCs were incubated in the presence of 10 μg/ml PHA and 10 ng/ml phorbol 12-myristate 13-acetate with TGFβ1 at four different concentrations (32 and 3.2 ng/ml, and 320 and 32 pg/ml) for 48 h. Supernatant was collected and measured for IL-4, IL-2 and IFNγ using ELISA. Figure 3 showed that TGFβ1 significantly inhibited IL-4 synthesis even at 320 pg/ml ($p = 0.004$). However, the effects of TGFβ1 on IL-2 and IFNγ production were not statistically significant (data not shown). The suppressing effect of TGFβ1 on cytokine production, especially of the Th2 type, may be involved in the association of the TGFβ1 genotype with reduced immunological activities.

Discussion

TGFβ belong to a super family of about 40 structurally related polypeptide growth factors [10]. Perturbation of the TGFβ superfamily has been implicated in various human diseases, e.g. cancer, angiogenesis, fibrotic diseases, and atherosclerosis, due to their mediation of many key events in normal growth and development including cellular differentiation, proliferation, motility, organization and death [11]. TGFβ1 is one of the best-studied factors in mammalian tissues. The role of TGFβ1 in asthma and allergic diseases has also been well documented in the literature [12]. Increased TGFβ1 expression in asthma has been repeatedly reported. Overexpression of TGFβ1 mRNA and proteins in bronchial biopsies from subjects with severe and moderate asthma has been observed compared with controls without asthma [13–15]. In asthmatic airways, TGFβ1 mRNA expression mainly produced from eosinophils was related to the degree of disease severity, subepithelial fibrosis and airway remodeling [13, 14, 16].

The genetics of atopy and asthma has been a very interesting research area in past years. Deciphering secrets in genetic variations related to allergy and asthma development may greatly benefit our efforts in the prevention and treatment of asthma and allergic diseases. The candidate gene approach, in which genes of relevance to the physiological changes of the disease process are tested for direct involvement with the disease, has been successfully applied to link genetic variation in the promoter region of a number of cytokine genes to asthma and allergic diseases. The promoter region of the TGFβ1 gene has been characterized showing regulatory impacts on transcriptional processes [17]. Our previous work demonstrated that a TGFβ1 promoter polymorphism at C-509T with the TT homozygote genotype is linked to allergic and asthmatic patients with elevated serum IgE [18]. Two case-control independent studies in relatively large populations of white individuals also found the association of

variant T allele with asthma, even with severity of asthma [5, 6, 19]. In our present study, the T variant of the genetic polymorphism TGFβ1 C-509T is significantly associated with allergy status. These results consistently support our hypothesis that the TGFβ1 T allele genotype is an important risk factor in allergy development.

C to T base change is located in the region of the promoter associated with Yin Yang-1 activator consensus sequence. It has been reported to increase promoter function using luciferase constructs [20]. The T allele has been demonstrated to enhance the YY1 binding site on the TGFβ1 promoter region and may account for the increased transcription of TGFβ [6]. The C-509T polymorphism was significantly associated with increased concentration of latent TGFβ1 in serum in a human study [21]. Our present results also showed an increased trend of plasma TGFβ1 to the T allele. Limited sample sizes and differences in population properties may explain the lack of statistical significance in this study. It is reasonable to deduce that subjects with the T allele may display a higher level of TGFβ1 in other tissues such as respiratory airways or skin. The observed overexpression of TGFβ1, e.g. in bronchial biopsies of asthmatic patients as discussed before, may at least partly result from their enhanced capacity to produce TGFβ1 determined by their genetic components such as the T allele mutation in C-509T, although a feedback mechanism from the inflammatory reaction in asthmatic patients cannot be excluded.

It has been well known that immunological activity plays a crucial role in initiating and developing allergic reactions in response to allergens. In mice, knockout studies with TGFβ1 indicated that TGFβ1 might be one of the most important growth and regulatory factors in the immune system because the knockout mice died of massive inflammatory lesions [22]. However, the evidences about its effects in the immune system seem confusing and contradictory. Its anti-inflammatory effect has been demonstrated by inhibiting the proliferation of T and B lymphocytes [23, 24]. On the other hand, TGFβ1 has been found to be a growth factor by inducing proliferation of macrophages, dendritic cells, and even T cells [25, 26]. Our present study indicates that a C to T mutation at the TGFβ1 genetic polymorphism C-509T is correlated with suppressed T-cell proliferation in vivo. This suggests that subjects with the T allele in the absence or presence of allergy demonstrated reduced lymphocyte response after exposure to allergen, even at the basic level. Increased production of TGFβ1 seems to be one of the reasons, since the plasma TGFβ1 concentration is negatively correlated with the degree of T-cell proliferation, and TGFβ1 suppresses T-cell proliferation in vitro at physiological concentrations. Human subjects with the T allele may own a relatively lower immune activity due to more TGFβ1 inside their body.

Allergic diseases are aberrant immune responses to antigen mainly mediated by IgE. Our previous study indicated that the C to T base exchange is associated with elevated IgE in atopic asthmatic patients. However, other studies did not confirm our findings [6]. Our present study failed to show a significant association with the total IgE level while other possible covariates have been considered. Lack of adjustment of covariates, e.g. the allergy status, in our previous report might be one reason for the elevated IgE in atopic asthma patients. These results implied a complicated relationship between TGFβ1 and total IgE. As we know, TGFβ1 is believed to inhibit IgE predominately in immunity. It might be related to its downregulatory effect on various cell surface molecules including CD23 and MHC class II antigen in human cells, which may be very important in promoting IgE synthesis [27, 28]. TGFβ1 could also inhibit e-germline transcription [29] directly through transcription factors or through inhibiting Th2 cytokines such as IL-4. Elevated TGFβ1 in TT allele subjects is expected to reduce the IgE level. However, as IgE synthesis is a very complicated process involving lots of cytokines (e.g. IL-13 and IL-10), even suppression of IgE is environment directed. It has been reported that at a later stage of differentiation, IgE B cells are refractory to the inhibitory effect of TGFβ, as shown by the slight but significant increase in the spontaneous secretion of IgE by PBMC of atopic patients [30]. Finally, TGFβ1 downregulation of IgE may be a mechanism where Th2 inflammation may still be prominent, with no increase in IgE, a situation that might be seen in intrinsic asthma for example. Therefore, we may not be surprised to see the lack of association of this genetic polymorphism with the IgE level in plasma in our present study.

Both T-cell proliferation and production of IgE and cytokines, including Th2 and Th1, are believed to play an important role in allergic inflammation. In our study, the Th2 cytokine IL-4 was depressed rather than IL-2 and IFNγ, implying that Th2 cytokines are crucial in explaining the observed suppressing effect of TGFβ1 on T-cell proliferation. However, the suppressing effect of TGFβ1 on the Th1-type reaction or initiation of T-cell reactions may still exist. This is because the TGFβ1-induced downregulation of the IL-2 receptor at both protein and mRNA level has been reported [29]. TGFβ1 has been shown to inhibit IL-2-induced tyrosine phosphorylation, activation of Jak-1 and Stat 5, and IL-2-induced expression of

α and β chains of IL-2R [30]. Interaction of IL-4 and IFNγ has been reported. Reduction of IL-4 by TGFβ1 may reduce the forces to inhibit IFNγ production. These may be some reasons that we failed to observe the significant effect on IFNγ. With respect to the cytokine IL-4, T-cell proliferation in the allergic reaction, an increased TGFβ1 concentration in T-allele subjects may play a role in the suppression of the allergic reaction or inflammatory effect rather than an increase in the risk of allergic status. The mechanisms involved are still not clear. More studies in larger patient cohorts and on the mechanisms regarding its role in allergic development may be worth of much more attention in the future. Other roles than cell proliferation for TGFβ1 may be hidden behind the association of TGFβ1 C-509T with allergy. As an example, TGFβ1 is an important stimulant of fibrosis, inducing formation of the extracellular matrix, and promoting wound healing and scar formation. Therefore, the profibrotic effect of TGFβ1 may play a more important role in asthma development. Another important function for TGFβ1 is their regulatory activity in cell differentiation. IL-3 and M-CSF in cooperation with TGFβ induces granulocyte macrophage colony-stimulating factor-independent differentiation of human CD34+ hematopoietic progenitor cells into dendritic cells with features of Langerhans' cells. TGFβ1 may play an important role in the differentiation and maturation of dendritic cells and monocyte differentiation. TGFβ1 may play an important role in regulating some cell cycle arrest rather than others, as indicated in CD34+ CD38-c-kit(high) vs. c-kit(low/–) cells. Due to the complexity in the development of allergic diseases, the T allele may be combined with other genetic variations, which produce a different environment leading to the allergic status.

In summary, our study demonstrated the modulatory effects of TGFβ1 and its polymorphism on cell proliferation and cytokine production. Further investigations with larger sample sizes could further solidify our findings. Clarification of the association of the TGFβ1 C-509T polymorphism with allergic diseases may raise new and better methods for diagnosis, and more importantly, the potential for new pharmacological interventions related to the products of the candidate genes.

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References

1 Burchard EG, Silverman EK, Rosenwasser LJ, Borish L, Yandava C, Pillari A, et al: Association between a sequence variant in the IL-4 gene promoter and FEV(1) in asthma. Am J Respir Crit Care Med 1999;160:919–922.

2 Nicolaides NC, Holroyd KJ, Ewart SL, Eleff SM, Kiser MB, Dragwa CR, et al: Interleukin 9: A candidate gene for asthma. Proc Natl Acad Sci USA 1997;94:13175–13180.

3 Lim S, Crawley E, Woo P, Barnes PJ: Haplotype associated with low interleukin-10 production in patients with severe asthma. Lancet 1998;352:113.

4 Chagani T, Pare PD, Zhu S, Weir TD, Bai TR, Behbehani NA, et al: Prevalence of tumor necrosis factor-alpha and angiotensin converting enzyme polymorphisms in mild/moderate and fatal/near-fatal asthma. Am J Respir Crit Care Med 1999;160:278–282.

5 Pulleyn LJ, Newton R, Adcock IM, Barnes PJ: TGFβ1 allele association with asthma severity. Hum Genet 2001;109:623–627.

6 Silverman ES, Palmer LJ, Subramaniam V, Hallock A, Mathew S, Vallone J, et al: Transforming growth factor-β1 promoter polymorphism C-509T is associated with asthma. Am J Respir Crit Care Med 2004;169:214–219.

7 Shull MM, Ormsby I, Kier AB, Pawlowski S, Diebold RJ, Yin M, et al: Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. Nature 1992;359:693–699.

8 Kehrl JH, Wakefield LM, Roberts AB, Jakowlew S, Alvarez-Mon M, Derynck R, et al: Production of transforming growth factor beta by human T lymphocytes and its potential role in the regulation of T cell growth. J Exp Med 1986;163:1037–1050.

9 Thompson MW, McInnes RR, Willard HF: Genetics in Medicine, ed 5. Philadelphia, Saunders, 1991.

10 Chang H, Brown CW, Matzuk MM: Genetic analysis of the mammalian transforming growth factor-beta superfamily. Endocr Rev 2002;23:787–823.

11 Blobe GC, Schiemann WP, Lodish HF: Role of transforming growth factor B in human disease. N Engl J Med 2000;342:1350–1358.

12 Duvernelle C, Freund V, Frossard N: Transforming growth factor-beta and its role in asthma (review). Pulm Pharmacol Ther 2003;16:181–196.

13 Vignola AM, Chanez P, Chiappara G, Merendino A, Pace E, Rizzo A, et al: Transforming growth factor-beta expression in mucosal biopsies in asthma and chronic bronchitis. Am J Respir Crit Care Med 1997;156:591–599.

14 Minshall EM, Leung DY, Martin RJ, Song YL, Cameron L, Ernst P, et al: Eosinophil-associated TGF-beta1 mRNA expression and airway fibrosis in bronchial asthma. Am J Respir Cell Mol Biol 1997;17:326–333.

15 Ohno I, Lea RG, Flanders KC, Clark DA, Banwatt D, Dolovich J, Denberg J, Harley CB, Gauldie J, Jordana M: Eosinophils in chronically inflamed human upper airway tissues express transforming growth factor B1 gene (TGF-β1). J Clin Invest 1992;89:1662–1668.

16 Ohno I, Nitta Y, Yamauchi K, Hoshi H, Honma M, Woolley K, et al: Transforming growth factor beta 1 (TGFβ1) gene expression by eosinophils in asthmatic airway inflammation. Am J Respir Cell Mol Biol 1996;15:404–409.

▶17 Kim SJ, Glick A, Sporn MB, Roberts AB: Characterization of the promoter region of the human transforming growth factor-beta 1 gene. J Biol Chem 1989;264:402–408.
▶18 Hobbs K, Negri J, Klinnert M, Rosenwasser LJ, Borish L: Interleukin-10 and transforming growth factor-beta promoter polymorphisms in allergies and asthma. Am J Respir Crit Care Med 1998;158:1958–1962.
▶19 Buckova D, Izakovicova Holla L, Benes P, Znojil V, Vacha J: TGF-beta1 gene polymorphisms. Allergy 2001;56:1236–1237.
▶20 Luedecking EK, DeKosky ST, Mehdi H, Ganguli M, Kamboh MI: Analysis of genetic polymorphisms in the transforming growth factor-β1 gene and the risk of Alzheimer's disease. Hum Genet 2000;106:565–569.
▶21 Grainger DJ, Heathcote K, Chiano M, Snieder H, Kemp PR, Metcalfe JC, et al: Genetic control of the circulating concentration of transforming growth factor type β1. Hum Mol Genet 1999;8:93–97.
▶22 Kulkarni AB, Huh CG, Becker D, Geiser A, Lyght M, Flanders KC, et al: Transforming growth factor beta 1 null mutation in mice causes excessive inflammatory response and early death. Proc Natl Acad Sci USA 1993;90:770–774.
▶23 Fargeas C, Wu CY, Nakajima T, Cox D, Nutman T, Delespesse G: Differential effect of transforming growth factor beta on the synthesis of Th1- and Th2-like lymphokines by human T lymphocytes. Eur J Immunol 1992;22:2173–2176.
▶24 Kehrl JH, Roberts AB, Wakefield LM, Jakowlew S, Sporn MB, Fauci AS: Transforming growth factor beta is an important immunomodulatory protein for human B lymphocytes. J Immunol 1986;137:3855–3860.
▶25 Fan K, Ruan Q, Sensenbrenner L, Chen B: Transforming growth factor-beta 1 bifunctionally regulates murine macrophage proliferation. Blood 1992;79:1679–1685.
▶26 Schiott A, Widegren B, Sjogren HO, Lindvall M: Transforming growth factor-β1, a strong costimulator of rat T-cell activation promoting a shift towards a Th2-like cytokine profile. Immunol Letters 1999;67:131–139.
▶27 Berg DJ, Lynch RG: Immune dysfunction in mice with plasmacytomas. I. Evidence that transforming growth factor-beta contributes to the altered expression of activation receptors on host B lymphocytes. J Immunol 1991;146:2865–2872.
▶28 Reimold AM, Kara CJ, Rooney JW, Glimcher LH: Transforming growth factor beta 1 repression of the HLA-DR alpha gene is mediated by conserved proximal promoter elements. J Immunol 1993;151:4173–4182.
▶29 Kehrl JH, Thevenin C, Rieckmann P, Fauci AS: Transforming growth factor-beta suppresses human B lymphocyte Ig production by inhibiting synthesis and the switch from the membrane form to the secreted form of Ig mRNA. J Immunol 1991;146:4016–4023.
▶30 Wu CY, Brinkmann V, Cox D, Heusser C, Delespesse G: Modulation of human IgE synthesis by transforming growth factor-beta. Clin Immunol Immunopathol 1992;62:277–284.

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Effects of dioxins on the quantitative levels of immune components in infants

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Dioxins (polychlorinated dibenzo-*p*-dioxin (PCDD)+polychlorinated dibenzofuran (PCDF)) and polychlorinated biphenyls (PCBs) are potentially hazardous compounds and have structural similarity with thyroid hormones. Animal studies have demonstrated that PCDDs, PCDFs and PCBs can alter immune functions. However, in humans it is not yet elucidated whether dioxins contained in breast milk have any effects on the immune functions in infants. To investigate the effects of dioxins on the immune system, we compared the quantitative levels of immune components between a breast-fed group and bottle-fed group, in which dioxin concentration is almost zero. Ratios of immune cells, such as CD4+ and CD8+ T-lymphocytes, as well as B-lymphocytes (CD19+ and/or CD20+) and NK cells (CD16+, CD56+) in peripheral blood lymphocytes, serum immunoglobulin level, and level of specific IgE antibody to allergens in the venous blood at 12 months of age were assessed in a subgroup of 281 infants. The relationship of post-natal dioxin exposure via breast feeding with the ratio of immunological markers and the level of humoral antibodies up to 12 month of age was not demonstrated. In conclusion, it would appear that the content of dioxins in breast milk in the Japanese general population is not enough to induce any change in theses-examined immunological parameters during the first year of life, although long-term effects remain to be evaluated. *Toxicology and Industrial Health* 2006; **22:** 131–136.

Key words: *breast feeding; bottle feeding; dioxins; IgE; lymphocytes subsets*

Introduction

Polychlorinated-dibenzo-*p*-dioxin (PCDD), polychlorinated-dibenzofuran (PCDF), and coplanar-polychlorinated biphenyl (Co-PCB) compounds, hereafter referred to as dioxins, are tricycle aromatic compounds. They are mainly formed as byproducts of the synthesis of organochlorine chemicals and from the combustion of municipal and hazardous waste. In the late 1970s, the production and use of these compounds were banned because their adverse health effects had become evident.

Immune suppression is a common and extensively characterized sequela associated with acute 2,3,7,8,-tetrachloro-dibenzo-*p*-dioxin (TCDD)

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exposure in laboratory animals. Comprehensive cell-type fractionation-reconstitution studies have previously demonstrated the profound inhibition of B-cell function by TCDDs. According to direct addition studies utilizing primary cultured murine B cells, there is evidence suggesting that the suppression of antibody production by TCDDs may be closely associated with altered B-cell differentiation (Suh *et al.*, 1983). This finding is further supported by the observation that TCDDs only modestly inhibit B-cell proliferation.

There is a paucity of *in vivo* studies on the effects of dioxins on the immune system of humans (Tryphona *et al.*, 1998). Initial studies, showing that PCB and dioxins may be toxic to human immune function, were carried out on individuals accidentally exposed to these compounds (Patterson *et al.*, 1988). Weisglas-Kuperus *et al.* (1995) demonstrated that prenatal PCB/dioxin exposure was associated with changes in T cell subpopulations in the blood in Dutch infants.

It is not yet clearly determined, however, whether pre- and post-natal exposures to high background levels of PCDD, PCDF and PCB can alter the immune system in human infants, and whether the health of infants is adversely affected by these pollutants. In this study, we investigated the effects of dioxins contained in breast milk on the quantitative levels of various immune components in Japanese infants from birth to 12 months of age.

Subjects and methods

We collected breast milk from 415 mothers in 20 prefectures and cities in Japan at 30 days post-partum and quantified 14 isomers for PCDDs, 15 for PCDFs and 12 for coplanar PCBs (Co-PCBs). To express the toxic potency of the mixture of dioxins in breast milk samples, the toxic equivalency (TEQ) calculation, based on the new TEF re-evaluated by WHO in 1997, was used. The ages of the mothers were limited to 25–34 years, and all mothers were primiparous and resided in the same area for more than five years.

At one year of age, blood samples were obtained from 281 breast-fed infants (breast-fed group) for the evaluation of immune functions. The breast-fed group was infants who had mainly received breast feeding until one year of age. Blood samples were also obtained from 20 infants who were bottle-fed at one year of age, as a control group (bottle-fed group).

The fat content in human milk was determined by weighing, as described by Patterson *et al.* (1988). In brief, breast milk (50 mL) was mixed with saturated potassium citrate (10 mL), ethanol (100 mL), diethylether (50 mL) and hexane (120 mL) in a 500-mL separatory funnel and shaken vigorously for 10 min. The hexane phase was then removed and washed first with 2 mol/L NaOH followed by sulfuric acid. The hexane phase was then dried and weighed. The fat content of breast milk at five days post-partum was $3.0 \pm 1.4\%$, and at 30 days post-partum was $3.8 \pm 1.2\%$, and did not change thereafter (Matsuura, 2001a,b).

PCDDs, PCDFs and Co-PCBs in human milk were identified by GC/MS conducted at the Japan Food Research Laboratory (Matsuura, 2001b). Surface markers of peripheral blood monocytes (PBMCs) were quantified by flow cytometry (SRL, Tokyo, Japan) (Ip *et al.*, 1982).

Serum IgE concentrations were determined by chemiluminescent enzyme immunoassay (Matsui *et al.*, 2000). Specific IgE antibodies for house dust, milk and egg white were quantified by fluoroenzyme assay (CAP RAST FEIA, Pharmacia & Upjohn, Sweden). All parents who participated in this study gave their written informed consent.

The ratio of CD3, CD4, CD8, CD4/8, CD19, CD20 and CD86 between the breast-fed and bottle-fed groups was analysed by Student's t-test. The serum immunoglobulin levels of IgG, IgA, IgM and IgE between the breast-fed and bottle-fed groups were analysed by Student's t-test. Distribution of CAP-RAST scores between 0 and 1–6 was analysed by Fisher's exact test. Probability (P) values <0.05 were considered to be statistically significant.

Results

Effect of dioxins in breast milk on T cell ratio in PBMCs

The ratios of CD3+, CD4+, CD8+ or CD4+/CD8+ cells in PBMCs were compared between the breast-fed and bottle-fed groups (Table 1). No significant differences were demonstrated. The correlation between the concentration of dioxins in human milk and T cell ratio was also investigated

Table 1. The ratio of lymphocyte subsets in the breast-fed and bottle-fed groups.

	Breast-fed group (N=281)	Bottle-fed group (N=20)	P
CD3 (%)	73.1±7.2	69.9±6.1	0.053
CD4 (%)	50.1±8.4	48.6±6.7	0.436
CD8 (%)	24.1±5.8	24.1±7.4	0.861
CD4/CD8	2.2±0.9	2.3±1.2	0.718
CD19 (%)	14.7±5.6	15.9±6.7	0.362
CD20 (%)	14.3±5.6	15.9±5.8	0.242
CD86 (%)	0.8±0.5	1.2±0.9	0.078
IgG (mg/dL)	645.1±182.1	694.0±186.2	0.248
IgA (mg/dL)	34.2±22.6	36.6±19.1	0.644
IgM (mg/dL)	105.9±33.6	106.9±41.4	0.899
IgE (U/mL)	54.4±89.9	58.2±105.9	0.857

and no significant correlation was found between them (Figure 1A).

Effect of dioxins in breast milk on B cell ratio in PBMCs

The ratios of CD19+, CD20+ or CD86+ cells, which are the surface markers of activated B cells, were compared between the breast-fed and bottle-fed groups (Table 1). There was no significant correlation. The correlation between the concentration of dioxin in human milk and B cell ratio was also investigated and no significant correlation was found between them (Figure 1A).

Effect of dioxins in breast milk on NK cell ratio in PBMCs

The correlation of the ratio of NK cells (CD16+/CD56+) with the concentration of dioxins in human milk was examined and no significant correlation was found (Figure 1A).

Effect of dioxins in breast milk on the serum immunoglobulin levels

The serum immunoglobulin levels of IgG, IgA, IgM and IgE were compared between the breast-fed and bottle-fed groups (Table 1). No significant differences were demonstrated between them. The correlation between the concentration of dioxins in human milk and the serum immunoglobulin levels was also investigated and no significant correlation was found between them (Figure 1B).

The specific IgE antibody to house dust, milk and egg white was quantified (Table 2). It was not demonstrated that there was no significant correlation between the breast-fed and bottle-fed groups.

Discussion

In this study, we investigated the relationship of the concentration of dioxins contained in breast milk with the ratios of immune cells and immunoglobulin levels. It has been reported that *in vitro* dioxins suppress B cell differentiation. Furthermore, there are some *in vivo* studies suggesting an effect of dioxins on immune functions (Forawi *et al.*, 2004). Smoger *et al.* (1993) reported that for children born to mothers living in a TCDDs-contaminated environment in Time Beach (MO) during and after pregnancy, a decrease in CD4+ T cells and an increase in CD8+ T cells was detected in children from nine to 14 years of age. In one preliminary study conducted in Northern Quebec, the CD4+/CD8+ T cell ratio of Inuit infants, whose mothers have increased levels of PCB and dioxins in their breast milk, decreased at six and 12 months of age (Dewailly *et al.*, 1993).

Svensson *et al.* (1994) reported that the consumption of fatty fish species, such as salmon and herring, from the Baltic Sea is an important source of human exposure to persistent organochlorine compounds, eg, PCDDs, PCDFs and co-PCBs. The high fatty-fish consumers had lower ratios and numbers of NK cells, identified by the CD56 marker, in peripheral blood than the non-consumers. The weekly intake of fatty fish correlated negatively with the ratio of NK cells. This indicates that accumulation of persistent organochlorine compounds in high fatty-fish consumers may adversely affect NK cell ratios. Weisglas-Kuperus *et al.* (1995) demonstrated that a high post-natal PCB/dioxin exposure is associated with an increase in the number of TcRγδ+ T cells at birth and with an increase in the number of CD8+, TcRαβ+ or TcRγδ+ T cells at 18 months of age. Nagayama *et al.* (1998) reported that the ratios of CD4+ to CD8+ T cells had a significant increasing tendency with the estimated total TEQ intakes.

In our study, it was not demonstrated that the ratios of T cell subpopulation and CD16+ CD56+ cells (NK cells) in PBMCs correlated with the concentration of dioxins in human milk, although the number of CD4+ and CD8+ T cells were not

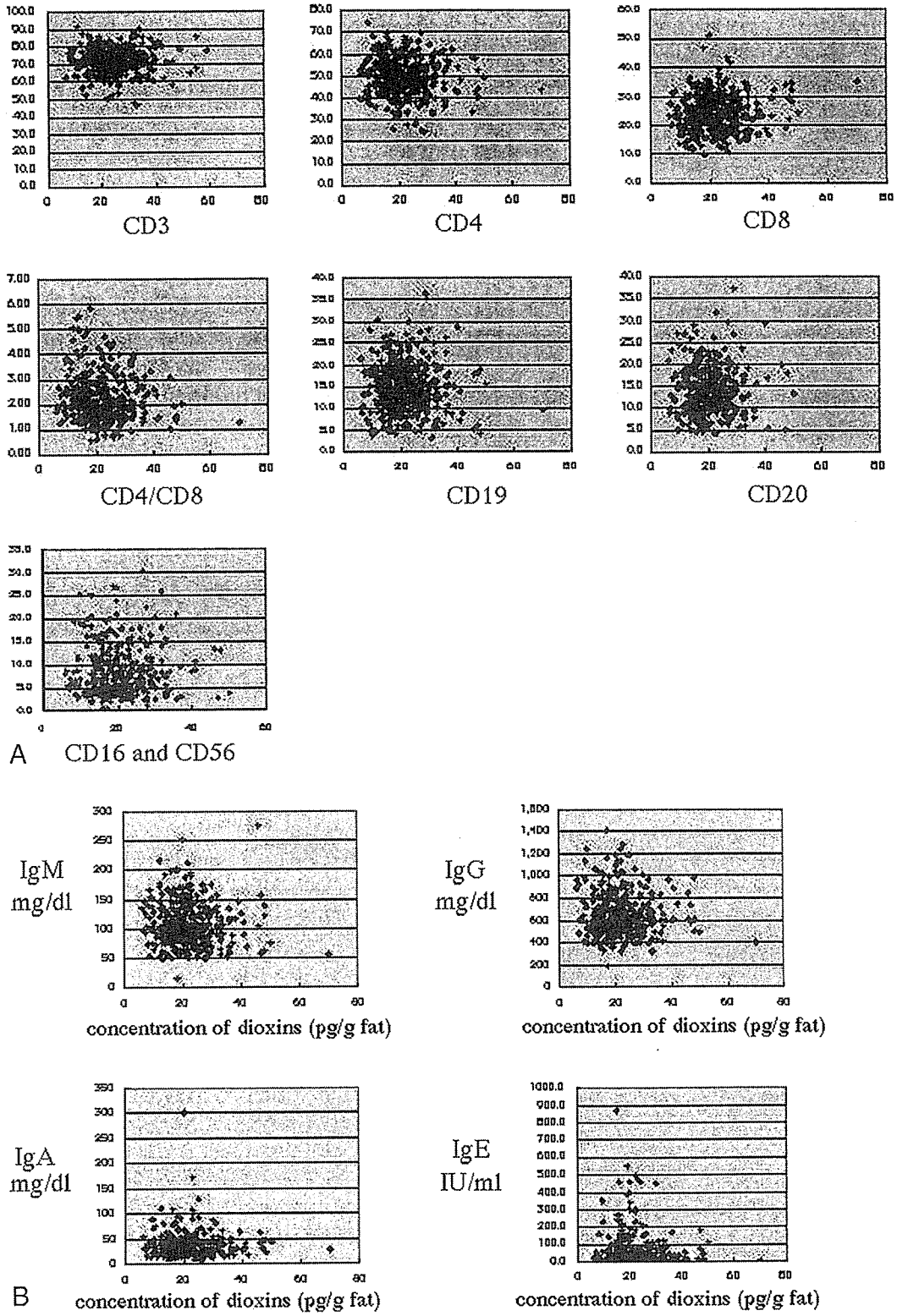

Figure 1. (A) Relationships between ratios of CD3+, CD4+, CD8+, CD4+/CD8+, CD19+, CD20+ and CD16+ CD56+ cells in PBMCs and the concentration of dioxins in human milk at 30 post-partum days. X-axis means the concentration of dioxins (pg/g fat). Y-axis means the percent of surface marker positive cells except CD4/CD8. In CD4/CD8, Y-axis means the ratio of CD4 per CD8. (B) Relationships between ratios of serum IgM, IgG, IgA and IgE and the concentration of dioxins in human milk at 30 post-partum days. X-axis means the estimated intake of dioxins (pg/g fat). Y-axis means the serum concentration of IgM (mg/dL), IgG (mg/dL), IgA (mg/dL) and IgE (IU/mL).

Table 2. Specific IgE antibody of breast-fed and bottle-fed groups.

	Breast-fed group							Bottle-fed group							*P*
	0[a]	1	2	3	4	5	6	0	1	2	3	4	5	6	
House dust	251[b]	8	10	5	1	1	0	19	0	1	0	0	0	0	0.568
Milk	238	12	20	5	1	0	0	17	0	2	1	0	0	0	0.558
Egg white	180	24	44	22	2	3	0	15	3	2	0	0	0	0	0.264

[a]Number (0–6) indicates CAP-RAST scores to each allergens.
[b]Number indicates the number of persons who have the CAP-RAST score.
Distribution of CAP-RAST scores between 0 and 1–6 was analysed by Fisher's exact test.

analysed. We could not find that the levels of serum immunoglobulins and specific IgE to allergen were significantly different between the breast-fed and bottle-fed groups. The difference between our data and those of Weisglas-Kuperus may be due to the following: the time at which immunological analysis was performed, and the amount of dioxins to which the subjects were exposed, that is, a higher concentration of dioxins in the early days after birth; the content of dioxins in breast milk was almost 2-fold higher in the Netherlands (30.75 pg TFQ/g fat) than in our study (14.8±6.1 pg TFQ/g fat) (Matsuura *et al.*, 2001b).

The sample size in this study was 281 in the breast-fed group and 20 in the bottle-fed group, which is a maximum size considering the budget for this study and the co-operation of the mothers. When we consider the value between the breast-fed and bottle-fed groups (shown in Table 1 as true difference), the power of CD3 and CD86 was higher than 50%, however, CD8, CD4/8, IgA, IgM and IgE was lower than 10%.

On the basis of the results of this study, we conclude that, although the infants were exposed to some amounts of dioxins in the breast milk in Japan, we could not find that the quantitative levels of immune components at one year of age was seriously impaired. However, long-term effects remain to be evaluated.

References

Dewailly, E., Bruneau, S. and Laliberte, C. *et al.* 1993: Breast milk contamination by PCBs and PCDDs/PCDFs in Arctic Quebeq: preliminary results on the immune status of Inuit infants. Short paper. In *Dioxin '93*. Presented at the Thirteenth International Symposium on Chlorinated Dioxins and Related Compounds, Vienna, 403–406.

Forawi, H.A., Tchounwou, P.B. and McMurray, R.W. 2004: Xenoestrogen modulation of the immune system: effects of dichlorodiphenyltrichloroethane (DDT) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). *Reviews on Environmental Health* 19, 1–13.

Ip, S.H., Rittershaus, C.W. and Healey, K.W. *et al.* 1982: Rapid enumeration of T lymphocytes by a flow-cytometric immunofluorescence method. *Clinical Chemistry* 28, 1905–909.

Matsui, E., Kaneko, H. and Teramoto, T. *et al.* 2000: Reduced IFNgamma production in response to IL-12 stimulation and/or reduced IL-12 production in atopic patients. *Clinical and Experimental Allergy* 30, 1250–56.

Matsuura, N., Uchiyama, T. and Tada, H. *et al.* 2001a: Effects of dioxins and polychlorinated biphenyls (PCBs) on thyroid function in infants born in Japan: report from research on environmental health. *Clinical Pediatrics Endocrinology* 10, 1–6.

Matsuura, N., Uchiyama, T. and Tada, H. *et al.* 2001b: Effects of dioxins and polychlorinated biphenyls (PCBs) on thyroid function in infants born in Japan – the second report from research on environmental health. *Chemosphere* 45, 1167–71.

Nagayama, J., Tsuji, H. and Iida, T. *et al.* 1998: Postnatal exposure to chlorinated dioxins and related chemicals on lymphocyte subsets in Japanese breast-fed infants. *Chemosphere* 37, 1781–87.

Patterson, D.G. Jr., Needham, L.L. and Pirkle, J.L. *et al.* 1988: Correlation between serum and adipose tissue levels of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in 50 persons from Missouri. *Archives of Environmental Contamination and Toxicology* 17, 139–43.

Smoger, G.H., Kahn, P.C. and Rodgers, G.C. *et al.* 1993: *In utero* and postnatal exposure to 2,3,7,8-TCDD in Times Beach, Missouri. 1. Immunological effects; lymphocytes phenotype frequencies. Short paper. In *Dioxin '93*. Presented at the Thirteenth International Symposium on Chlorinated Dioxins and Related Compounds, Vienna, pp. 345–48.

Suh, J., Jeon, Y.J. and Kim, H.M. *et al.* 1983: Aryl hydrocarbon receptor-dependent inhibition of AP-1 activity by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in activated B cells. *Toxicology and Applied Pharmacology* 68, 434–41.

Svensson, B.G., Hallberg, T. and Nilsson, A. *et al.* 1994: Parameters of immunological competence in subjects with high consumption of fish contaminated with persistent

organochlorine compounds. *International Archives of Occupational and Environmental Health* 65, 351–58.

Tryphona, H. 1998: The impact of PCBs and dioxins on children's health: immunological considerations. *Canadian Journal of Public Health* 89, S49–52.

Weisglas-Kuperus, N., Sas, T.C.J. and Koopman-Esseboom, C. *et al*. 1995: Immunological effects of background prenatal and postnatal exposure to dioxins and polychlorinated biphenyls in Dutch infants. *Pediatric Research* 38, 404–10.

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PEDIATRIC ALLERGY AND IMMUNOLOGY

Suppression of IFN-gamma production in atopic group at the acute phase of RSV infection

Kaneko H, Matsui E, Asano T, Kato Z, Teramoto T, Aoki M, Kawamoto N, Lian LA, Kasahara K and Kondo N. Suppression of IFN-gamma production in atopic group at the acute phase of RSV infection. Pediatr Allergy Immunol 2006: 17: 370–375.
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Several studies have suggested that respiratory syncytial virus (RSV) bronchiolitis induced the change of cytokine production profile in childhood. We sought to determine whether the RSV-induced cytokine production was affected by the patient's atopic background. We quantified interferon-gamma (IFN-gamma) and interleukin (IL)-4 in the supernatant of peripheral blood mononuclear cells (PBMCs) cultured for 24 h and in the presence of phytohemaglutinin (PHA), IL-12, or IL-18, from 14 infants who were divided into two groups, those who are non-atopic and an atopic group. In RSV-infected infants with atopic diseases, IFN-gamma production from IL-12- or especially IL-18-stimulated PBMCs was subtotally suppressed in the acute phase, whereas in RSV-infected infants without atopic diseases IFN-gamma production was not suppressed on acute phase. The IFN-gamma suppression observed in the atopic group is not caused by the immaturity of an infant's immune system since reduced IFN-gamma production to RSV is not observed in the infants of non-atopic group. IFN-gamma suppression in regard to RSV infection might be caused by some genetic factor involved in the development of atopic disease such as IL-18 signal cascade.

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Respiratory syncytial virus (RSV) remains the most prevalent infectious cause of lower respiratory tract illness in both infants and children (1–3). Most infections with RSV are symptomatic; however, the range of severity in infants and young children varies greatly. Several studies have suggested that infants in whom RSV bronchiolitis develops will have recurrent wheezing and asthma later in childhood (4). The reasons why this occurs are still unknown; whether RSV is the actual causal factor for asthma or simply targets children who are predisposed to bronchial obstructive disease is still speculation (5). Further complicating the picture is the question of whether there is a link between RSV infection in infancy and the development of wheeze or asthma later in childhood. Results from clinical studies have been contradictory, with some results supporting a link, and others disproving a link (6, 7).

It has been shown in animal studies that viral infections can increase the risk of allergic sensitization. A viral influence causing a switch from Th2 to Th1 lymphocytes, and thus a lower production of IgE antibodies, has been suggested, but a more complex mechanism may operate in RSV infection, judging from animal studies and the results of the present and other clinical studies (8). It has been hypothesized that an early RSV infection may perpetuate Th2-dominant immune responses (9).

Aberle et al. tried to compare both the levels of IFN-gamma mRNA expression in peripheral blood mononuclear cells (PBMCs) and the distribution of lymphocyte subpopulations in

infants with severe RSV bronchiolitis and those with a milder clinical course of illness (10). Their data indicated that reduced IFN-gamma expression might be an important factor in the pathogenesis of severe RSV disease in infancy.

In this study we tried to determine whether lymphocytes in children with atopy or some genetic background show a different immune response to RSV compared with non-atopic children. Our results showed that the reduced IFN-gamma expression was observed specifically in the atopic group.

Subjects and methods

Subjects

Fourteen children between 1 month and 4 yr of age, were admitted for respiratory tract infection to our hospital during the epidemic period of 2002–2003. All of our cases were admitted in our hospital according to the dyspnea and/or the exhaustion because of the continuance of the high fever. They had infections with a fever and coughing with and without bronchial obstruction. Their nasopharyngeal secretions were positive for the RSV antigen by enzyme-linked immunosorbent assay (ELISA) kit (Directigen RS, Becton, Japan). They were divided into two groups: (i) with no allergic disease or history thereof, and (ii) with allergic disease or a family history and/or a past history of allergic disease. The atopic group in this study consisted of subjects with bronchial asthma and atopic dermatitis or a family history within one generation of allergic diseases. There were no significant differences in age, sex and clinical severity between the non-atopic and atopic group. The diagnosis of bronchial asthma was made according to the criteria of the American Thoracic Society, and that of atopic dermatitis was made according to the criteria of Hanifin. Informed consent was obtained from their parents. All of the subjects were randomly selected among patients in our hospital. This study was approved by Gifu University ethical committee.

Cell preparation and culture

Peripheral blood mononuclear cells were isolated from the heparinized blood of patients by gradient centrifugation in Ficoll-Paque (Pharmacia AB, Uppsala, Sweden). PBMCs were suspended to give a density of 10^6/ml in the culture medium which consisted of RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, L-glutamine (2 mmol/l), penicillin (100 U/ml) and streptomycin (100 μg/ml). PBMCs (10^6/ml) were cultured in the presence of 10 μg/ml phytohemagglutinin (PHA) (Gibco BRL, Grand Island, NY, USA), interleukin (IL)-12 (5 IU/ml), or IL-18 (400 ng/ml) in a volume of 1 ml in a round-bottom tube (Falcon 2059, Becton Dickinson Labware, Lincoln Park, NJ, USA) at 37°C in a humidified atmosphere containing 5% CO_2. The recombinant IL-18 had been prepared in our laboratory through use of an *Escherichia coli* expression system [according to a method developed by Kato et al. (11)].

Assays for IFN-gamma and IL-4

The test-tube culture supernatants were spun to remove the cells after culturing and were stored, frozen at −80°C until assayed. IFN-gamma concentrations were measured with a human IFN-gamma ELISA kit (Ohtsuka, Tokyo, Japan), and the detection limit was 15.6–1000 pg/ml. IL-4 concentrations of the culture supernatants were measured with a human IL-4 US ELISA kit (Bio Source, Camarillo, CA, USA). When cytokine values were not detectable, for statistical analysis the minimum detectable level was used.

IgE assay

Plasma samples obtained from heparinized blood were kept at −30°C. Plasma IgE concentrations were determined by chemiluminescent enzyme immunoassay. The values were regarded as the serum IgE concentrations. Specific IgE antibodies for house dust and *Dermatofagoides farinae* were determined by fluoloenzyme assay (CAP RAST FEIA, Pharmacia & Upjohn, Sweden).

Statistical analysis

The significance of the difference between the control and atopic group was analyzed by the Wilcoxon Mann Whitney test. A statistical significance was assumed for p-values < 0.05.

Results

The patients with RSV infection were divided into non-atopic and atopic group (Table 1). Clinical severity was determined by the period of fever, wheezing and the hospitalization period. No patients had a prior episode to RSV infection. There were no significant differences in age, sex and clinical severity although the hospitalization period of atopic group seemed to be longer than non-atopic group ($p = 0.079$). In all