Hardy-Weinberg equilibrium. This did not result in significant difference in the genotype frequency between cases and controls. The data suggest that case samples were not well representative of the homogeneous population. We genotyped another set of 94 cases with this SNP. Genotypes CC, CT, and TT were 25, 50, and 19 cases respectively. Genotype frequency of the new set of patients was in Hardy-Weinberg equilibrium and not significantly different from the control value. Although we cannot exclude *AICDA* completely as a candidate gene for asthma, the effect of the 465C/T polymorphism on susceptibility to asthma was not as strong as the repeat polymorphisms of *STAT6* or *NOS1*.

In conclusion, we demonstrated that *STAT6* and *NOS1* loci are associated with childhood asthma in the Japanese population and showed evidence of "suggestive" linkage between region 12q24.23–q24.33 and asthma. This chromosome region most likely contains as yet unidentified asthma susceptibility gene(s).

Acknowledgement We thank all members of the families and volunteers who participated in this study for their kind cooperation. We also thank Ms. Kumi Kato for excellent technical assistance. This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology, Japan, and grants from the Ministry of Health, Labor, and Welfare, Japan.

References

Barnes KC, Neely JD, Duffy DL, Freidhoff LR, Breazeale DR, Schou C, Naidu RP, Levett PN, Renault B, Kucherlapati R, Iozzino S, Ehrlich E, Beaty TH, Marsh DG (1996) Linkage of asthma and total serum IgE concentration to markers on chromosome 12q: evidence from Afro-Caribbean and Caucasian populations. Genomics 37:41–50

Barnes KC, Freidhoff LR, Nickel R, Chiu YF, Juo SH, Hizawa N, Naidu RP, Ehrlich E, Duffy DL, Schou C, Levett PN, Marsh DG, Beaty TH (1999) Dense mapping of chromosome 12q13.12-q23.3 and linkage to asthma and atopy. J Allergy Clin Immunol 104:485–491

Collaborative Study on the Genetics of Asthma (CSGA) (1997) A genome-wide search for asthma susceptibility loci in ethnically diverse populations. Nat Genet 15:389–392

Daniels SE, Bhattacharrya S, James A, Leaves NI, Young A, Hill MR, Faux JA, Ryan GF, le Souef PN, Lathrop GM, Musk AW, Cookson WO (1996) A genome-wide search for quantitative trait loci underlying asthma. Nature 383:247–50

Dizier MH, Besse-Schmittler C, Guilloud-Bataille M, Annesi-Maesano I, Boussaha M, Bousquet J, Charpin D, Degioanni A, Gormand F, Grimfeld A, Hochez J, Hyne G, Lockhart A, Luillier-Lacombe M, Matran R, Meunier F, Neukirch F, Pacheco Y, Parent V, Paty E, Pin I, Pison C, Scheinmann P, Thobie N, Vervloet D, Kauffmann F (2000) Genome screen for asthma and related phenotypes in the French EGEA study. Am J Respir Crit Care Med 162:1812–1818

Duetsch G, Illig T, Loesgen S, Rohde K, Klopp N, Herbon N, Gohlke H, Altmueller J, Wjst M (2002) STAT6 as an asthma candidate gene: polymorphism-screening, association and haplotype analysis in a Caucasian sib-pair study. Hum Mol Genet 11:613–621

Ferris BG (1978) Epidemiology Standardization Project (American Thoracic Society). Am Rev Respir Dis 118:1–120

Fujii K, Matsubara Y, Akanuma J, Takahashi K, Kure S, Suzuki Y, Imaizumi M, Iinuma K, Sakatsume O, Rinaldo P, Narisawa K (2000) Mutation detection by TaqMan-allele specific amplification: application to molecular diagnosis of glycogen storage disease type Ia and medium- chain acyl-CoA dehydrogenase deficiency. Hum Mutat 15:189–196

Gao PS, Kawada H, Kasamatsu T, Mao XQ, Roberts MH, Miyamoto Y, Yoshimura M, Saitoh Y, Yasue H, Nakao K, Adra CN, Kun JF, Moro-oka S, Inoko H, Ho LP, Shirakawa T, Hopkin JM (2000a) Variants of NOS1, NOS2, and NOS3 genes in asthmatics. Biochem Biophys Res Commun 267:761–763

Gao PS, Mao XQ, Roberts MH, Arinobu Y, Akaiwa M, Enomoto T, Dake Y, Kawai M, Sasaki S, Hamasaki N, Izuhara K, Shirakawa T, Hopkin JM (2000b) Variants of STAT6 (signal transducer and activator of transcription 6) in atopic asthma. J Med Genet 37:380–382

Grasemann H, Yandava CN, Storm van's Gravesande K, Deykin A, Pillari A, Ma J, Sonna LA, Lilly C, Stampfer MJ, Israel E, Silverman EK, Drazen JM (2000) A neuronal NO synthase (NOS1) gene polymorphism is associated with asthma. Biochem Biophys Res Commun 272:391–394

Haagerup A, Bjerke T, Schiotz PO, Binderup HG, Dahl R, Kruse TA (2002) Asthma and atopy—a total genome scan for susceptibility genes. Allergy 57:680–686

Hakonarson H, Bjornsdottir US, Halapi E, Palsson S, Adalsteinsdottir E, Gislason D, Finnbogason G, Gislason T, Kristjansson K, Arnason T, Birkisson I, Frigge ML, Kong A, Gulcher JR, Stefansson K (2002) A major susceptibility gene for asthma maps to chromosome 14q24. Am J Hum Genet 71:483–491

Heinzmann A, Grotherr P, Jerkic SP, Lichtenberg A, Braun S, Kruse S, Forster J, Kuehr J, Deichmann KA (2000a) Studies on linkage and association of atopy with the chromosomal region 12q13–24. Clin Exp Allergy 30:1555–1561

Heinzmann A, Mao X, Akaiwa M, Kreomer RT, Gao P, Ohshima K, Umeshita R, Abe Y, Braun S, Yamashita T, Roberts MH, Sugimoto R, Arima K, Arinobu Y, Yu B, Kruse S, Enomoto T, Dake Y, Kawai M, Shimazu S, Sasaki S, Adra CN, Kitaichi M, Inoue H, Yamauchi K, Tomichi N, Kurimoto F, Hamasaki N, Hopkin JM, Izuhara K, Shirakawa T, Deichmann KA (2000b) Genetic variants of IL-13 signalling and human asthma and atopy. Hum Mol Genet 9:549–559

Hyden C, Pereira E (1997) Mutation screening of interferon gamma (IFN gamma) as a candidate gene for asthma. Clin Exp Allergy 27:1412–1416

Immervoll T, Loesgen S, Dutsch G, Gohlke H, Herbon N, Klugbauer S, Dempfle A, Bickeboller H, Becker-Follmann J, Ruschendorf F, Saar K, Reis A, Wichmann HE, Wjst M (2001) Fine mapping and single nucleotide polymorphism association results of candidate genes for asthma and related phenotypes. Hum Mutat 18:327–336

Ioannidis JP, Ntzani EE, Trikalinos TA, Contopoulos-Ioannidis DG (2001) Replication validity of genetic association studies. Nat Genet 29:306–309

Isidoro-García M, Rosa-Gómez S, Davila I, Lorente F, Gonzalez-Sarmiento R (2003) Lack of association between the 7888 C/T polymorphism in the *AID* gene and atopy in a Spanish population. J Allergy Clin Immunol 112:460–461

Kruglyak L, Lander E (1995) Complete multipoint sib-pair analysis of qualitative and quantitative traits. Am J Hum Genet 57:439–454

Kruglyak L, Daly MJ, Reeve-Daly MP, Lander ES (1996) Parametric and nonparametric linkage analysis: a unified multipoint approach. Am J Hum Genet 58:1347–1363

Laitinen T, Daly MJ, Rioux JD, Kauppi P, Laprise C, Petays T, Green T, Cargill M, Haahtela T, Lander ES, Laitinen LA, Hudson TJ, Kere J (2001) A susceptibility locus for asthma-related traits on chromosome 7 revealed by genome-wide scan in a founder population. Nat Genet 28:87–91

Lander E, Kruglyak L (1995) Genetic dissection of complex traits: guidelines for interpreting and reporting linkage results. Nat Genet 11:241–247

Malerba G, Lauciello MC, Scherpbier T, Trabetti E, Galavotti R, Cusin V, Pescollderungg L, Zanoni G, Martinati LC, Boner AL, Levitt RC, Pignatti PF (2000) Linkage analysis of chromosome 12 markers in Italian families with atopic asthmatic children. Am J Respir Crit Care Med 162:1587–1590

Mao XQ, Shirakawa T, Yoshikawa T, Yoshikawa K, Kawai M, Sasaki S, Enomoto T, Hashimoto T, Furuyama J, Hopkin JM, Morimoto K (1996) Association between genetic variants of mast-cell chymase and eczema. Lancet 348:581–583

Nakao F, Ihara K, Kusuhara K, Sasaki Y, Kinukawa N, Takabayashi A, Nishima S, Hara T (2001) Association of IFN-gamma and IFN regulatory factor 1 polymorphisms with childhood atopic asthma. J Allergy Clin Immunol 107:499–504

Nickel R, Wahn U, Hizawa N, Maestri N, Duffy DL, Barnes KC, Beyer K, Forster J, Bergmann R, Zepp F, Wahn V, Marsh DG (1997) Evidence for linkage of chromosome 12q15-q24.1 markers to high total serum IgE concentrations in children of the German Multicenter Allergy Study. Genomics 46:159–162

Noguchi E, Shibasaki M, Inudou M, Kamioka M, Yokouchi Y, Yamakawa-Kobayashi K, Hamaguchi H, Matsui A, Arinami T (2001) Association between a new polymorphism in the activation-induced cytidine deaminase gene and atopic asthma and the regulation of total serum IgE levels. J Allergy Clin Immunol 108:382–386

Ober C, Cox NJ, Abney M, Di Rienzo A, Lander ES, Changyaleket B, Gidley H, Kurtz B, Lee J, Nance M, Pettersson A, Prescott J, Richardson A, Schlenker E, Summerhill E, Willadsen S, Parry R (1998) Genome-wide search for asthma susceptibility loci in a founder population. The Collaborative Study on the Genetics of Asthma. Hum Mol Genet 7:1393–1398

Ober C, Tsalenko A, Parry R, Cox NJ (2000) A second-generation genomewide screen for asthma-susceptibility alleles in a founder population. Am J Hum Genet 67:1154–1162

O'Connell JR, Weeks DE (1998) PedCheck: a program for identification of genotype incompatibilities in linkage analysis. Am J Hum Genet 63:259–266

Raby BA, Silverman EK, Lazarus R, Lange C, Kwiatkowski DJ, Weiss ST (2003) Chromosome 12q harbors multiple genetic loci related to asthma and asthma-related phenotypes. Hum Mol Genet 12:1973–1979

Tamura K, Arakawa H, Suzuki M, Kobayashi Y, Mochizuki H, Kato M, Tokuyama K, Morikawa A (2001) Novel dinucleotide repeat polymorphism in the first exon of the STAT-6 gene is associated with allergic diseases. Clin Exp Allergy 31:1509–1514

Wilkinson J, Grimley S, Collins A, Thomas NS, Holgate ST, Morton N (1998) Linkage of asthma to markers on chromosome 12 in a sample of 240 families using quantitative phenotype scores. Genomics 53:251–259

Wjst M, Fischer G, Immervoll T, Jung M, Saar K, Rueschendorf F, Reis A, Ulbrecht M, Gomolka M, Weiss EH, Jaeger L, Nickel R, Richter K, Kjellman NI, Griese M, von Berg A, Gappa M, Riedel F, Boehle M, van Koningsbruggen S, Schoberth P, Szczepanski R, Dorsch W, Silbermann M, Wichmann HE, et al. (1999) A genome-wide search for linkage to asthma. German Asthma Genetics Group. Genomics 58:1–8

Xu J, Postma DS, Howard TD, Koppelman GH, Zheng SL, Stine OC, Bleecker ER, Meyers a D (2000) Major genes regulating total serum immunoglobulin E levels in families with asthma. Am J Hum Genet 67:1163–1173

Xu J, Meyers DA, Ober C, Blumenthal MN, Mellen B, Barnes KC, King RA, Lester LA, Howard TD, Solway J, Langefeld CD, Beaty TH, Rich SS, Bleecker ER, Cox NJ (2001a) Genomewide screen and identification of gene-gene interactions for asthma-susceptibility loci in three U.S. populations: collaborative study on the genetics of asthma. Am J Hum Genet 68:1437–1446

Xu X, Fang Z, Wang B, Chen C, Guang W, Jin Y, Yang J, Lewitzky S, Aelony A, Parker A, Meyer J, Weiss ST (2001b) A genomewide search for quantitative-trait loci underlying asthma. Am J Hum Genet 69:1271–1277

Yokouchi Y, Nukaga Y, Shibasaki M, Noguchi E, Kimura K, Ito S, Nishihara M, Yamakawa-Kobayashi K, Takeda K, Imoto N, Ichikawa K, Matsui A, Hamaguchi H, Arinami T (2000) Significant evidence for linkage of mite-sensitive childhood asthma to chromosome 5q31-q33 near the interleukin 12 B locus by a genome-wide search in Japanese families. Genomics 66:152–160

Identification of granulocyte subtype–selective receptors and ion channels by using a high-density oligonucleotide probe array

Toshiharu Nakajima, PhD,[a,b] Motoyasu Iikura, MD, PhD,[c] Yoshimichi Okayama, MD, PhD,[d] Kenji Matsumoto, MD, PhD,[a,c] Chitose Uchiyama, MD,[c] Taro Shirakawa, MD,[e] Xing Yang, PhD,[f] Chaker N. Adra, PhD,[f,g] Koichi Hirai, MD, PhD,[c] and Hirohisa Saito, MD, PhD[a,d]

Tokyo, Kyoto, and Yokohama, Japan, and Boston, Mass

Background: During inflammation, neutrophils, basophils, and eosinophils release cell type–specific mediators and proteases through signaling molecules, such as G protein–coupled receptors and ion channels. As such, ion channels and receptors, including G protein–coupled receptors, are common drug targets.

Objective: We sought to identify, for the first time, ion channels and receptors preferentially expressed by each granulocyte subtype.

Methods: Using GeneChip, we compared approximately 20,000 transcripts present in 7 leukocyte types, platelets, mast cells, and fibroblasts to identify granulocyte subtype–selective transcripts for receptors and ion channels. Granulocyte subtype–selective transcripts were chosen on the basis of several conditions, such as the transcript having a 5-fold or greater expression level compared with the maximum level of other leukocytes.

Results: Fifty-one transcripts were chosen to be preferentially expressed by each granulocyte subtype. Seventeen of the 51 transcripts have not been previously reported as granulocyte subtype selective. Among the 17 receptors and ion channels, 6 were basophil selective, eosinophil selective, or both and were not highly expressed by other organs, indicating that they might be potential targets for antiallergy drugs.

Conclusion: Use of this database of potential cell type–selective drug targets should minimize the efforts required for pharmaceutical development. (J Allergy Clin Immunol 2004;113:528-35.)

Key words: Basophils, eosinophils, granulocytes, G protein–coupled receptors, ion channels

Three types of human blood granulocytes, eosinophils, basophils, and neutrophils, play roles in protecting against microbial infection by releasing cell type–specific mediators and proteases. Specifically, eosinophils and basophils evoke allergic reactions and damage nematodes.[1,2] As well as killing bacteria, neutrophils sometimes induce systemic vasculitis or multiple organ damage under certain conditions.[3,4] Thus targeting granulocyte type–selective functions is considered an important strategy for drug discovery.

Activation of blood granulocytes and tissue mast cells is generally characterized by an influx of extracellular calcium (Ca^{2+}), which is essential for subsequent release of granule-derived mediators, newly generated lipid mediators, and cytokines.[5] The mechanism by which granulocyte mediator secretion is sustained is therefore likely to include modulation of various types of ion channels. Flow of ions, including K^+ and Cl^-, might play an important role during granulocyte responses because they regulate cell membrane potential and thus influence Ca^{2+} influx.[6] Treatment of mast cells and basophils with pertussis toxin inactivates the Gi type of G proteins and abolishes degranulation induced by nonimmunologic ligands, such as thrombin and N-formylpeptide; however, it fails to inhibit the influx of Ca^{2+}.[7] Thus Ca^{2+}-independent stimulation of Gi is also involved in granulocyte degranulation. The thrombin (protease)–activated receptors and formylpeptide receptors are classified as G protein–coupled receptors (GPRs), having an extracellular N-terminal segment, a 7-transmembrane region that forms the transmembrane core, 3 exoloops, 3 cytoloops, and a C-terminal segment.[8] Thus ion channels and GPRs both play essential roles in degranulation, as well as other cellular functions important for granulocytes. As a result, both ion channels and GPRs are targets of drug development.[9]

As the human genome project nears completion, the identification of potential drug targets by using gene expression profiles from specific cell types is becoming practical and important for drug discovery.[10,11] The

From [a]the Department of Allergy and Immunology, National Research Institute for Child Health and Development, Tokyo; [b]The Organization for Pharmaceutical Safety and Research, Tokyo; [c]the Department of Bioregulatory Function, University of Tokyo, Graduate School of Medicine, Tokyo; [d]the Laboratory for Allergy Transcriptome, RIKEN Research Center for Allergy and Immunology, Yokohama; [e]the Department of Health Promotion and Human Behavior, Kyoto University Graduate School of Public Health, Kyoto; [f]the Department of Medicine and Pathology, Beth Israel Deaconess Medical Center, Boston; and [g]the Department of Medicine, Children's Hospital, Harvard Medical School, Boston.

Supported in part by a grant from the Organization for Pharmaceutical Safety and Research and the Ministry of Health, Labour and Welfare (the Millennium Genome Project, MPJ-5), a grant from RIKEN Research Center for Allergy and Immunology (to H.S.), National Institutes of Health grant AI 43663 from the National Institute of Allergy and Infectious Diseases, grant RSG-01-241-01-LIB from the American Cancer Society (to C.A.), and by the Adra family.

Received for publication September 30, 2003; revised December 6, 2003; accepted for publication December 12, 2003.

Reprint requests: Hirohisa Saito, MD, PhD, Department of Allergy and Immunology, National Research Institute for Child Health and Development, 3-35-31 Taishido, Setagaya-ku 154-8567 Tokyo, Japan, or Chaker N. Adra, PhD, Department of Medicine, Beth Israel Deaconess Medical Center, 99 Brooklin Ave., Boston, MA 02215.

0091-6749/$30.00

© 2004 American Academy of Allergy, Asthma and Immunology

doi:10.1016/j.jaci.2003.12.036

Abbreviations used

AD: Average difference (of mRNA expression level)
Ca^{2+}: Extracellular calcium
EMR: Epidermal growth factor–like module containing mucin-like receptor
GAPDH: Glyceraldehyde-phosphate dehydrogenase
GPR: G protein–coupled receptor
PAR: Proteinase-activated receptor
Siglec: Sialic acid–binding Ig-like lectin

sequencing of the human genome is offering an unprecedented opportunity for the pharmaceutical development of drugs. Receptor genes and ion channel genes are found only in 5% and 1.3% of all genes present in the human genome, respectively.[10] However, receptors and ion channels are found in 45% and 5% of the molecular targets of all known drugs, respectively.[9,12,13] Thus receptors, including GPRs, and ion channels are now considered the most important drug targets.

Until recently, it has been impractical to analyze genome-wide expression of leukocytes. A newly developed technology, the microarray or high-density oligonucleotide probe array (GeneChip), is one of the latest breakthroughs in experimental molecular biology, which allows approximately 39,000 transcripts derived from a cell's transcriptome to be simultaneously monitored. Using this technology, we previously reported the transcriptome profiling of various types of mast cells and eosinophils.[14-16] In this study we used GeneChip (version U133A, containing approximately 22,000 gene probes) to examine the cell type–selective transcriptome expression of 7 types of leukocytes (basophils, eosinophils, neutrophils, $CD4^+$ cells, $CD8^+$ cells, $CD14^+$ cells, and $CD19^+$ cells), platelets, mast cells, and fibroblasts. Then we focused on the expression of granulocyte-selective genes for ion channels, GPRs, and other receptors. We identified 17 novel granulocyte subtype–selective transcripts that might prove useful for drug development.

METHODS

Purification of leukocytes

All human subjects in this study provided written informed consent, and the ethical review boards at the relevant hospitals (National Center for Child Health and Development and Jikei University School of Medicine) approved the study. The subjects used in this study were all healthy volunteers, specifically chosen for having no allergic diseases.

Granulocytes and mononuclear cells were separated from the venous blood of normal volunteers. Human basophils were semipurified by means of Percoll (Pharmacia, Uppsala, Sweden) density gradient centrifugation, and the cells were further purified by means of negative selection through use of a MACS Basophil Isolation Kit (Miltenyi Biotech, Bergisch-Gladbach, Germany), as described previously.[17] Eosinophils were isolated by using Percoll (1.090 g/mL) density centrifugation. The eosinophils were further purified by means of negative selection with anti-CD16–bound micromagnetic beads, as described previously.[18] Neutrophils were isolated by using Percoll (1.085 g/mL) density centrifugation and further purified by means of negative selection with anti-CD81 antibody and anti-mouse IgG-bound micromagnetic beads to eliminate contaminating eosinophils. These granulocytes purified from human peripheral blood were spun down onto glass slides by using the Cytospin II (Shandon Southern Instruments Inc, Sewickley, Pa). The purity of these cells was evaluated on the basis of 500 cells stained with May-Grünwald and Giemsa solutions.

For preparation of lymphocytes and monocytes, PBMCs were isolated by means of centrifugation on lymphocyte separation medium (Organon Teknika Corp, Durham, NC). Monocytes ($CD14^+$ cells) were prepared with magnetic bead–conjugated $CD14^+$ antibody (CD14 MicroBeads; Miltenyi Biotec) from PBMCs. $CD4^+$ and $CD8^+$ cells were also sorted by using magnetic bead–conjugated $CD4^+$ (CD4 MicroBeads; Miltenyi Biotec) and $CD8^+$ antibodies (CD8 MicroBeads; Miltenyi Biotec), respectively, from PBMCs after depletion of $CD14^+$ cells with MACS CD14 MicroBeads (Miltenyi Biotec). The purity of $CD4^+$, $CD8^+$, and $CD14^+$ cells was evaluated by staining the magnetic bead–conjugated cells compared with feasible control cell preparations, such as unpurified cells, with FITC-labeled goat anti-mouse Ig (BD Pharmingen, Tokyo, Japan). Peripheral B cells were purified by using a combination of negative (MicroBead-conjugated antibodies to CD3, CD7, CD14, CD42b, and CD56; Miltenyi Biotec) and positive (CD19 MicroBeads; Miltenyi Biotec) selection with MicroBeads (Miltenyi Biotec). Blood samples were mixed with 3.8% (wt/vol) sodium citrate solution (9:1) and centrifuged at 260*g* for 15 minutes at 20°C to obtain platelet-rich plasma. The plasma was centrifuged again at 260*g* for 15 minutes to remove any contaminating erythrocytes and leukocytes.

Human mast cells were derived from cord blood $CD34^+$ progenitor cells, as described previously.[11-14] Briefly, progenitor cells purified from peripheral blood by using $CD34^+$ isolation kits (Miltenyi Biotec) were cultured in Iscove modified Dulbecco medium supplemented with 1% insulin-transferrin-selenium supplements (Invitrogen, Tokyo, Japan), 50 μmol/L 2-mercaptoethanol, antibiotics, and 2% FCS in the presence of 100 ng/mL stem cell factor and 50 ng/mL IL-6. After 11 to 14 weeks of culture, tryptase-positive cells represented more than 99% of the cells.

Purification of human lung mast cells and nasal polyp–derived fibroblasts

Normal human lung tissue dissected during surgery was obtained macroscopically after obtaining informed consent. Human lung mast cells were dispersed from chopped lung specimens by means of an enzymatic procedure and were purified by using magnetic bead affinity selection with the mAb anti-kit YB5.B8 (BD PharMingen, San Diego, CA), as described previously.[19] The cells were further cultured in the presence of stem cell factor and IL-6 for several weeks. Human nasal polyp–derived fibroblasts were obtained as previously reported.[20]

GeneChip expression analysis

Human genome–wide gene expression was examined by using the Human Genome U133A probe array (GeneChip; Affymetrix, Santa Clara, Calif), which contains the oligonucleotide probe set for 22,000 full-length genes. Experiments were performed in accordance with the manufacturer's protocol (Expression Analysis Technical Manual) and previous reports.[11-14] Total RNA (1-5 μg) was extracted from 10^7 cells. Double-stranded cDNA was synthesized by using a SuperScript Choice system (Invitrogen) and a T7-(dT)24 primer (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom). The cDNA was subjected to in vitro transcription in the presence of biotinylated nucleoside triphosphates by using a BioArray high-yield RNA transcript labeling kit (Enzo Diagnostics, Farmingdale, NY). The biotinylated cRNA was hybridized with a

probe array for 16 hours at 45°C. In some experiments, as indicated in the supplementary table, biotinylated cRNA was prepared by using 2 cycles of cDNA synthesis, and in vitro transcription for target amplification was performed according to the manufacturer's directions (Affymetrix, Inc). For the latter protocol, we used 100 ng of total RNA. After washing, the hybridized biotinylated cRNA was stained with streptavidin-phycoerythrin (Molecular Probes, Eugene, Ore) and then scanned with an HP gene array scanner. The fluorescence intensity of each probe was quantified by using a computer program, GeneChip Analysis Suite 5.0 (Affymetrix). The expression level of single mRNA was determined as the average fluorescence intensity among the intensities obtained with 11 paired (perfect-matched and single nucleotide-mismatched) probes. If the intensities of mismatched probes were very high, gene expression was judged to be absent, even if a high average fluorescence was obtained with the GeneChip Analysis Suite 5.0 program. The level of gene expression was determined as the average difference (AD) by using the GeneChip software. Each AD level was then normalized by dividing it with the median value of 22,283 AD levels obtained in an experiment (ie, the normalized AD level).

Real-time RT-PCR

Total RNA was isolated with Isogen (Nippon gene, Tokyo, Japan), according to the manufacturer's instructions, and quantified by measuring the absorbance at 260 nm. RNA was subsequently treated with DNase I (Invitrogen) reverse transcribed by using Superscript II reverse transcriptase (Invitrogen). Real-time RT-PCR was performed with 10 ng of cDNA in 25 µL of final volume by using the primers and probes supplied by the Assays-on-Demand Gene Expression System (Applied Biosystem, Tokyo, Japan), according to the manufacturer's instructions. Measurement of gene expression was performed with the ABI PRISM 7700 Sequence Detector. The expression level of each gene was normalized to a reduced glyceraldehyde-phosphate dehydrogenase (GAPDH).

Staining of basophils with anti-HTm4

Basophils purified from human peripheral blood with the Basophil Isolation Kit (Miltenyi Biotec) were spun down onto glass slides by using the Cytospin II (Shandon). Cells were fixed with aceton for 1 minute and then blocked in 2% goat serum in 50 mmol/L Tris-Cl, pH 7.4, for 1 hour. Cells were further incubated for 2 hours with 2 µg/mL of the polyclonal antibody rabbit anti-hHTm4. Cells were then washed 3 times with PBS and incubated with a secondary antibody, highly cross-adsorbed Alexa Fluor 546-conjugated goat anti-rabbit IgG (H+L; Red; Molecular Probes), for 1 hour. After 3 PBS washes, air-dried cells were further mounted by using the Prolong Anti-Fade Kit (Molecular Probes). Slides were scanned with a Zeiss Laser Scanning Microscope 5 Pascal (Carl Zeiss Microimaging Inc, Thornwood, NY).

Purity and viability of the leukocytes and RNA quality

We used leukocyte samples in this study only if the purity of each cell type was at least 98%, but there were greater than 0.5% contaminated cells in any of the samples. We could not evaluate the purity of CD19⁺ cells and platelets because of the lack of feasible controls or methods. However, specific transcript markers for nongranulocytes (eg, CD4, CD8, CD14, CD19, and IgG), as well as granulocyte subtype-specific transcripts, were reasonably expressed by each leukocyte type, as shown in Online Repository Table E1. Regarding the viability, we qualified the RNA before GeneChip assay by using Array Quality Metrics Comparisons Software (Affymetrix), as well as trypan blue staining (they were always >95% viable), because RNAse-rich granules derived from degenerating cells rapidly destroy RNA transcripts. Briefly, to evaluate the quality of RNA, the ratio of the 3′-probe set and 5′-probe set of housekeeping genes were compared as shown in Online Repository Table E1. According to the above software's guidance, the ratio of greater than 2:1 at a standard sample (5 µg total RNA) protocol and that of greater than 10:1 at a small sample (50 ng of total RNA) protocol were recommended. As shown in Online Repository Table E1, the cells used in the present study had the appropriate ratios of the 3′-probe set and 5′-probe set of housekeeping genes, suggesting that these cells were highly viable.

Statistical analysis

Because logarithmic normalized AD levels were normally distributed within each group, the unpaired, parametric, 2-tailed Student *t* test was used to analyze the data on a logarithmic scale.

RESULTS

In this study we have used a high-density oligonucleotide probe array (GeneChip) to measure the expression levels of approximately 20,000 different transcripts in highly purified cells. These cells were basophils, eosinophils, neutrophils, monocytes ($CD14^+$), T lymphocytes ($CD4^+$ and $CD8^+$ cells), B lymphocytes ($CD19^+$), lung-derived mast cells, cord blood–derived cultured mast cells, and nasal polyp–derived fibroblasts. The GeneChip assay allows the simultaneous measurement of large numbers of transcripts by using relatively small numbers of cells. Using this technology, we could even measure triplicate transcriptome levels of basophils, the most rare granulocytes in peripheral blood.

Cell type–selective transcripts were selected on the basis of the following criteria. First, the average normalized AD expression level of each gene in a certain cell type must be 3-fold or greater than the maximal level in other cell types, and second, it must be significantly ($P < .01$) greater than that in other cell types. Third, the AD expression level provided with "absence" or "marginal" call by GeneChip Suite Software should be observed only once or not at all in the 3 or 4 independent experiments (3 experiments for basophils and 4 experiments for eosinophils and neutrophils) by using different cell populations. Finally, for the transcripts preferentially expressed for the 2 different cell types, such as basophils and eosinophils, the average normalized AD expression levels in the 2 cell types should be within 3-fold of each other. By using these standards, we found 83 basophil-selective, 37 eosinophil-selective, 257 neutrophil-selective, 34 basophil/eosinophil-selective, 19 eosinophil/neutrophil-selective, and 17 basophil/neutrophil-selective transcripts. Because of the functional similarity with basophils, mast cell–selective transcripts were also examined, and 63 mast cell–selective and 11 mast cell/basophil–selective transcripts were also detected (Table I and Online Repository Table E1). Because mast cells, basophils, and eosinophils play similar roles in allergic inflammation, the transcripts preferentially expressed for the 3 granulocytes by comparing their average normalized AD levels with those of other leukocytes. Thirty-four transcripts were then selected; however, most of them were overlapped with the transcripts listed in

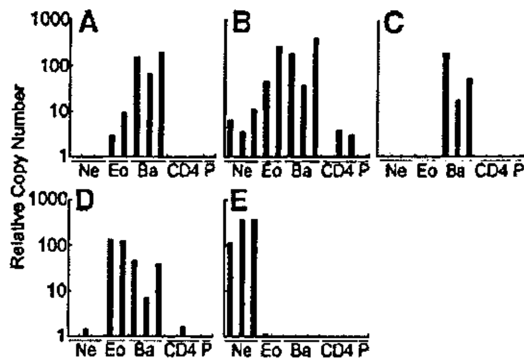

FIG 1. Real-time quantitative PCR analysis. The relative mRNA expression level by each cell type against PBMCs was shown after normalization of mRNA levels for HTm4 (0.42 per 100 GAPDH; A), Ca^{2+} receptor α 1D (0.003 per 100 GAPDH; B), prostaglandin E receptor 3a2 (0.12 per 100 GAPDH; C), EMR-1 (0.62 per 100 GAPDH; D), and aquaporin 9 (0.92 per 100 GAPDH; E), expressed by PBMCs. *Ne*, Neutrophils; *Eo*, eosinophils; *Ba*, basophils; *CD4*, $CD4^+$ cells; *P*, PBMCs.

Online Repository Table E1. Only 4 transcripts (*MYB*, *SAMSN1*, *BACE2*, and *CASP3*) were found not to be overlapped, and they were not receptors or ion channels.

Among the 491 granulocyte-selective transcripts listed in Online Repository Table E1, 4 ion channels, 19 GPRs, and 28 other receptors were further selected (Table II). When plural transcripts obtained by different probe sets had identical Genebank or Unigene accession numbers (http://www.ncbi.nlm.nih.gov/), the transcript showing the highest expression level was selected.

Ion channels and receptors preferentially expressed by granulocytes other than neutrophils

Eosinophils, basophils, and mast cells play an important role in the pathogenesis of allergic diseases but do not play an essential role in killing microbes, except for nematodes. On the other hand, neutrophils play a crucial role in killing microbes, such as bacteria. Caution should be taken in regulating neutrophil function, even in the case of neutrophil-induced inflammation. Therefore the molecules present only in granulocytes, except for neutrophils, would be important pharmaceutical targets for allergic disorders.[1,2]

Among the 51 granulocyte-selective transcripts for ion channels and receptors, we identified 17 granulocyte-selective transcripts that have not been reported for their selective expression (shown in bold letters in Table II). Of these 17 transcripts, 8 were preferentially expressed by granulocytes other than neutrophils. Among these 8 transcripts, the 2 transcripts for fibroblast growth factor receptor 2 and low-density lipoprotein receptor were found to be expressed by multiple tissue cell types (shown at http://www.lsbm.org/index_e.html, which displays genomic expression of 55 different human tissue cells, such as brain, heart, and lung cells, using the same experimental system [Affymetrix U133A] as ours). Thus they might not be suitable as a drug target because important organs that are unrelated to allergic inflammation (eg, the brain) express it. Among the 6 novel transcripts found to be preferentially expressed by granulocytes except for neutrophils, we focused on the following 4 transcripts expressed by granulocytes, including basophils. They were Ca^{2+} channel (*CACNA1D*), prostaglandin E receptor (*EP3A2*), epidermal growth factor–like module–containing mucin-like receptor (EMR) 1 (*EMR1*), and HTm4 (*MS4A3*).

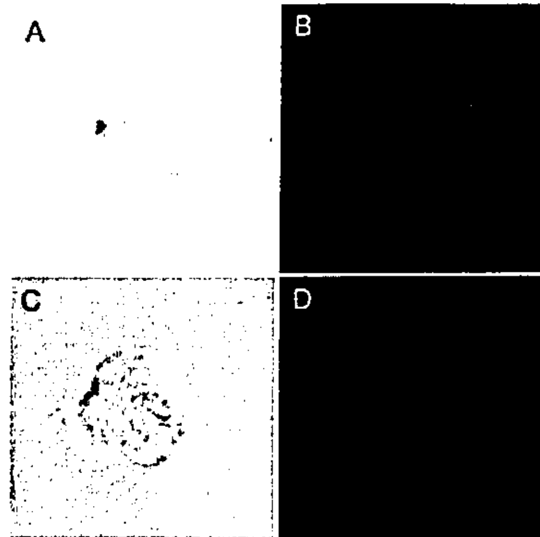

FIG 2. Demonstration of HTm4 protein on human basophils. Cells were incubated with 2 µg/mL polyclonal rabbit anti-hHTm4 antibody (A = phase contrast, B) or 2 µg/mL rabbit IgG (H+L) as a control (C = phase contrast, D), followed by incubation with a secondary antibody, highly cross-adsorbed Alexa Fluor 546–conjugated goat anti-rabbit IgG (H+L; Fig 2, B and D).

Basophils are the rarest granulocytes present in human peripheral blood, and as such, their complete transcriptional profiles remain unclear, and no basophil-selective transcripts have previously been reported.[21] Although eosinophils and mast cells have been considered important therapeutic targets for allergic diseases for a long time, recent studies suggest the importance of basophils in the pathogenesis of severe allergic diseases, such as fatal asthma.[2,22] Therefore we further examined the selective expression of these 4 basophil- or basophil/eosinophil-selective transcripts by using real-time RT-PCR. As shown in Fig 1, including a known molecule preferentially expressed by neutrophils, aquaporin 9,[23] the results obtained with the GeneChip assay were confirmed by using this highly accurate and reproducible method.[24]

Among these 4 molecules, we could obtain a suitable antibody against HTm4, which is a member of a family of 4-transmembrane proteins that includes CD20 and the FcεRI β-chain.[25] Genetics provided evidence for the existence of multiple loci relevant to atopic asthma on chromosome 11q13, including HTm4.[26] Most recently, we have published data identifying HTm4 as a hematopoietic cell-cycle regulator.[27] Using specific antibody against HTm4, we could detect the expression of HTm4 at the protein level in basophils (Fig 2). The

TABLE I. Representative cell type–selective transcripts in granulocytes

Accession no.*	Transcript	SI†	Normalized AD level
Basophil selective			
NM_000589.1	IL-4	73.3	13.3
L35848.1	HTm4	38.2	132.1
BC005912.1	FcεRIα	12.7	219.0
Eosinophil selective			
NM_001140.1	Arachidonate 15-lipoxygenase	74.1	18.3
NM_024703.1	FLJ22593	19.1	29.1
NM_014442.1	Siglec-8	9.8	16.9
Neutrophil selective			
NM_004633.1	IL-1R, type II	127.9	51.5
U73191.1	Inward rectifier K^+ channel Kir1.3	107.5	98
NM_001557.1	CXCR2 (IL-8Rβ)	39.3	105.2
Mast cell selective			
AF206667.1	Tryptase β	84.3	159.4
NM_001911.1	Cathepsin G	51.5	72.1
BC005929.1	Major basic protein	31.6	72.5
Basophil/eosinophil selective			
M75914.1	IL-5Rα	42.8	19.4 (B), 29.3 (E)
NM_004778.1	CRTH2	16.6	23.9 (B), 38.1 (E)
NM_001828.3	Charcot-Leyden crystal protein	15.2	229.2 (B), 198.6 (E)
Eosinophil/neutrophil selective			
NM_005306.1	GPR 43 (PAR1-like)	21.7	11.7 (E), 32.9 (N)
NM_004668.1	DHHC domain containing 18	6.6	16.2 (E), 44.5 (N)
Basophil/neutrophil selective			
NM_016006.1	CGI-58 protein	5.8	12.6 (B), 21.2 (N)
Basophil/mast cell selective			
NM_001870.1	Carboxypeptidase A3	59.2	111.7 (M), 137.3 (B)
NM_002529.2	TRK neurotrophin receptor	34.7	3.1 (M), 7 (B)
NM_000139.1	FcεRI β	21.2	22.2 (M), 43.8 (B)

*GenBank accession number (http://www.ncbi.nlm.nih.gov). †The selectivity index *(SI)* was calculated by comparing the normalized AD level in a cell type or the normalized AD level of 2 cell types with the maximal gene expression level of the other 8 or 9 cell types. The complete list of the genes having an SI of greater than 3 is shown in Online Repository Table E1.

amount of molecules expressed by various cell types should be quantified, and the effect of any identified antagonists should be tested on the cell types found to express these molecules to confirm whether these ion channels and receptors could be potential drug targets for diseases involving basophil activation.

As has been well documented and expected, IL-3 receptors, IL-5 receptors, chemokine receptor CCR3,[1,2] sialic acid–binding Ig-like lectin (Siglec)–8,[28] Siglec-6,[15] histamine H4 receptor,[29] and chemoattractant receptor–homologous molecule expressed on T_H2 cells (CRTH2)[30] were preferentially expressed by basophils, eosinophils, or both or mast cells. These consistent observations strengthened the reliability of the present methods and research strategy. Indeed, the antibody against Siglec-8 can induce selective apoptosis of eosinophils and is expected to be useful therapeutically.[28]

Ion channels and receptors preferentially expressed by granulocytes, including neutrophils

Of the 17 transcripts that have not been reported as granulocyte selective, 9 were preferentially expressed by granulocytes, including neutrophils. Pharmaceutical targets of selective granulocyte transcripts should treat inflammatory diseases without affecting the function of important organs that are unrelated to inflammation, as well as the function of lymphocytes. However, 4 of the 9 neutrophil-selective transcripts were expressed by multiple organ tissues. One of the 4 neutrophil-selective transcripts encoded proteinase-activated receptor (PAR)–2, a receptor for mast cell tryptase that is linked to the pathogenesis of allergic diseases, such as asthma.[31,32] PAR-2 transcripts are also abundantly expressed by tissue types, including skin and intestine, which are often the target organs for allergic diseases. But the development of PAR-2 antagonists for use as antiallergic drugs might be unsuitable because it might downregulate neutrophil function and thereby induce bacterial infection.

DISCUSSION

We identified 51 granulocyte-selective genes for ion channels and receptors by examining approximately 20,000 kinds of transcripts derived from 16,000 genes from 10 different types of cells with the U133A GeneChip, which covers approximately half of the genes present in the human genome. The majority of these transcripts encoded molecules known or expected to be granulocyte subtype–selective, such as the IL-3 receptor and Fcε receptors.

TABLE II. Granulocyte subtype–selective transcripts for ion channels and receptors

Transcript (GenBank accession no.)	Cell type*	MC	Ba	Eo	Ne	Pl	CD4	CD8	CD14	CD19	Fb	TC†
Ion channels												
Ca^{2+} channel type A1 D (BE550599)	Ba, Eo	0.1	1.7	1.5	0.4	0.0	0.3	0.1	0.0	0.3	0.0	*0*
Aquaporin 9 (NM_020980.2)	Ne	0.7	0.1	0.4	137.4	0.4	0.9	0.1	8.6	0.1	0.2	*7*
K^+ channel Kir1.3 (U73191.1)	Ne	0.9	0.2	0.4	99.5	0.7	0.2	0.0	0.8	0.0	0.0	*5*
K^+ channel Kir2.1 (AF153820.1)	Ne	0.7	3.8	5.3	40.7	0.4	0.2	0.5	1.5	0.7	1.1	*6*
GPR												
Histamine H_4 R (AF312230.1)	Ba	0.7	34.2	9.4	0.7	0.4	0.8	0.5	0.6	0.0	0.1	*0*
PGE R type 3a2 (X83858.1)	Ba	0.8	10.3	0.1	0.7	0.6	0.3	0.2	0.2	0.5	1.7	*0*
C3a R (U62027.1)	Ba, Eo	11.8	55.7	39.4	2.0	1.6	1.6	1.5	3.0	0.6	0.5	*3*
CCR3 (NM_001837.1)	Ba, Eo	0.6	117.4	90.9	24.9	0.2	0.5	0.4	0.2	0.2	0.4	*0*
CRTH2 (NM_004778.1)	Ba, Eo	1.1	26.0	38.2	2.0	0.8	1.4	1.0	1.2	0.9	0.5	*0*
EMR-1 (NM_001974.1)	Ba, Eo	0.8	33.5	90.9	4.2	3.4	1.7	0.8	7.1	1.6	0.5	*1*
Adenosine A_3 R (NM_000677.2)	Eo	2.6	2.9	15.4	2.3	1.2	1.6	0.5	2.0	0.5	0.5	*0*
P2Y2 purinergic R (NM_002564.1)	Eo	0.1	0.1	5.5	0.1	0.2	0.1	0.3	1.2	0.2	0.1	*0*
GPR105 purinergic R (NM_014879.1)	Eo	2.6	2.9	15.4	2.3	1.2	1.6	0.5	2.0	0.5	0.5	*2*
GPR, Edg-4 (AF011466.1)	Eo, Ne	1.3	2.8	15.9	24.2	0.1	3.6	5.0	3.8	1.9	0.9	*2*
PAR1-like GPR43 (NM_005306.1)	Eo, Ne	0.3	0.7	12.4	35.2	0.8	0.1	0.1	0.7	0.4	0.1	*0*
C5a R (NM_001736.1)	Ne	2.3	21.6	13.6	92.6	2.3	1.2	0.5	25.6	1.0	0.4	*5*
CXCR1 IL-8R (NM_000634.1)	Ne	0.2	4.3	0.3	83.4	0.4	0.3	0.2	0.2	0.1	0.1	*0*
CXCR2 IL-8R (NM_001557.1)	Ne	0.2	1.1	1.5	112.1	2.7	0.3	0.8	0.7	0.6	0.0	*1*
Formyl peptide R 1 (NM_002029.1)	Ne	2.8	23.5	8.8	282.9	3.6	1.3	0.7	62.6	1.1	0.5	*0*
Formyl peptide R 2 (U81501.1)	Ne	0.4	0.5	0.6	75.5	0.1	1.0	0.6	6.0	0.8	0.1	*5*
GPR77 (NM_018485.1)	Ne	0.1	0.9	0.8	3.4	0.0	0.3	0.2	1.0	0.1	0.3	*2*
GPR86 purinergic R (NM_023914.1)	Ne	0.3	0.2	17.9	88.2	1.9	0.1	0.2	12.3	0.6	0.0	*1*
PAR2 (BE965369)	Ne	0.1	0.3	2.2	36.2	0.1	0.7	0.1	1.6	0.4	1.2	*15*
Other receptors (R)												
FcεRIα (BC005912.1)	Ba	19.4	219.0	4.6	5.5	1.4	5.1	0.8	1.4	0.4	0.2	*0*
HTm4 (L35848.1)	Ba	0.6	133.1	6.9	3.5	0.2	0.5	0.4	0.1	0.2	0.0	*1*
IL-3 R (NM_002183.1)	Ba	0.6	52.6	2.0	0.7	0.2	0.3	0.2	0.7	0.1	0.1	*0*
CD244 NK cell R (NM_016382.1)	Ba, Eo	0.3	56.0	16.5	1.2	0.1	0.4	3.5	5.2	1.1	0.1	*0*
Fibroblast growth factor R 2 (NM_022969.1)	Ba, Eo	0.1	27.9	12.1	0.2	0.1	0.1	0.2	0.1	0.1	1.0	*17*
IL-5R α (M75914.1)	Ba, Eo	0.9	20.5	30.4	0.6	0.1	0.1	0.1	0.1	0.2	0.1	*0*
Siglec-8 (NM_014442.1)	Eo	1.8	0.3	17.4	0.4	0.3	0.2	0.1	0.2	0.5	0.2	*0*
CD117 c-KIT (NM_000222.1)	MC	89.0	7.2	4.2	1.2	0.8	0.2	0.5	0.1	0.1	0.5	*15*
Siglec-6 (D86358.1)	MC	5.6	0.2	0.0	0.2	0.5	0.0	0.0	0.0	0.4	0.0	*0*
FcεRIβ (NM_000139.1)	MC, Ba	22.3	44.3	0.4	0.6	1.6	0.5	0.2	0.0	0.3	0.1	*0*
Low-density lipoprotein R (NM_000527.2)	MC, Ba	20.7	20.4	1.3	1.8	2.3	2.1	3.4	3.5	0.8	6.3	*34*
Tropomyosin-related kinase neurotrophin R (NM_002529.2)	MC, Ba	4.6	7.3	0.1	0.1	0.1	0.1	0.1	0.1	0.0	0.0	*0*
Butyrophilin-like R (AK025267.1)	Ne	1.1	1.7	1.5	6.9	1.5	1.7	1.6	0.9	1.5	1.3	*1*
CD120a, TNF-R-I (NM_001065.1)	Ne	1.7	1.2	7.8	74.7	1.3	2.2	5.9	17.4	0.6	16	*35*
CD95, Fas, APO-1 (AA164751)	Ne	1.4	10.9	7.3	50.4	1.3	5.6	4.8	3.6	2.6	9.7	*7*
Decoy R1, TRAILR3 (AF012536.1)	Ne	0.1	1.5	7.7	78.7	0.5	0.2	0.1	0.7	0.1	0.6	*0*
FcγRIIc2 (U90939.1)	Ne	1.5	2.4	7.8	59.9	0.3	0.2	0.1	7.2	2.3	0.2	*2*
FcγRIIc3 (U90940.1)	Ne	2.4	10.7	10.0	84.3	3.0	1.4	0.6	14.0	7.2	1.0	*5*
FcγRIII (J04162.1)	Ne	0.7	1.6	1.9	199.6	6.6	1.3	1.3	2.2	2.6	0.1	*4*
G-CSF R (NM_000760.1)	Ne	0.1	0.4	1.6	163.6	0.2	0.8	0.2	25.5	0.1	0.2	*3*
IL-13R (U81379.3)	Ne	0.3	0.2	2.0	14.1	0.4	0.4	0.3	2.9	1.3	1.3	*0*
IL-1R, type II (NM_004633.1)	Ne	0.1	0.1	0.1	53.5	0.2	0.4	0.1	0.2	0.0	0.0	*3*
Insulin-like growth factor R1 (NM_000875.2)	Ne	0.3	3.5	5.0	17.4	1.6	0.1	2.8	2.4	2.0	3.5	*3*
Insulin-like growth factor R2 (NM_000876.1)	Ne	4.6	0.9	5.4	85.3	1.7	2.9	8.7	8.4	4.8	15	*32*
Leukocyte Ig-like R A2 (NM_006866.1)	Ne	0.5	5.8	4.3	41.2	1.8	0.0	0.1	11.6	0.5	0.1	*2*
Toll-like R1 (AL050262.1)	Ne	0.6	0.3	1.2	31.5	1.6	0.8	0.7	3.0	1.5	0.3	*0*
Toll-like R2 (NM_003264.1)	Ne	0.9	6.0	1.3	83.8	1.6	1.3	0.1	26.3	0.9	0.4	*0*
Toll-like R6 (NM_006068.1)	Ne	0.5	1.0	0.9	8.8	0.1	0.9	0.9	2.1	1.2	0.6	*0*

MC, Mast cells; *Ba,* basophils; *Eo,* eosinophils; *Ne,* neutrophils; *Pl,* platelets; *Fb,* fibroblasts; *TC,* tissue cells; *R,* receptor.

*Cell-type selectivity was obtained by comparing the normalized AD levels of each gene in mast cells, basophils, eosinophils, neutrophils, platelets, $CD4^+$ cells, $CD8^+$ cells, $CD14^+$ cells, $CD19^+$ cells, and fibroblasts.

†The expression of each gene by various tissue cells was determined by searching http://www.lsbm.org/index_e.html. The italicized numeral indicates the number of tissues that have a raw AD level of greater than 200.

Mast cells expressed low levels of FcεRIα compared with basophils, and even neutrophils expressed a substantial level of the receptor (Table II). This raises the possibility that the GeneChip assay might not be suitable for detecting selective molecules. In the present study, however, only the GeneChip data obtained by using cord blood–derived mast cells and lung mast cells could be used because of the strict data selection based on the RNA quality (see the "Methods" section). As has been reported,[14,33] peripheral blood–derived cultured mast cells or skin-derived mast cells expressed approximately 10-fold FcεRIα mRNA compared with that produced by cord blood–derived mast cells (data not shown). Also, as shown in Online Repository Table E1, only 2 of 4 neutrophil samples expressed FcεRIα mRNA. This might be explained by the observation that only neutrophils obtained from some allergic donors express the molecule.[34]

We unexpectedly found 17 granulocyte-selective transcripts, including HTm4. Basophil-selective transcripts, eosinophil-selective transcripts, or both identified in our study could be potential therapeutic targets for allergic diseases because these granulocytes play a crucial role in allergic inflammation.[1,2] Granulocyte-selective transcripts could also be drug targets for other inflammatory diseases, such as systemic vasculitis.[3,4] Analysis of cell type–selective transcripts from database searches is expected to minimize the efforts required for drug discovery. The public database (http://www.lsbm.org/index_e.html) shows that some granulocyte-selective transcripts (18 of 51) detected in our study are abundantly expressed by multiple (>3) organ tissue cell types by using the same GeneChip U133A probe array. Thus the safety of any candidate drug must be evaluated by comparing its efficacy (on granulocytes) with its toxicity (to organs). Six of the 17 novel granulocyte-selective molecules could be excluded from drug development because of their expression in multiple organs unrelated to the diseases. Thus our approach has identified 11 receptors and ion channels with therapeutic potential. Among the 11 receptors and ion channels, 7 were basophil selective, eosinophil selective, or both and were not expressed by other organs, indicating that they might be potential targets for antiallergic drugs.

Finally, it should be stressed that basophils, the rarest leukocytes, have recently been found to play a more crucial role than we ever proposed in the pathogenesis of intractable allergic diseases, such as fatal asthma.[35,36] Thus targeting basophil receptors and ion channels, such as HTm4 and the Ca^{2+} channel *CACNA1D*, is particularly expected for future drug discovery. The importance of molecules known to be expressed by basophils might be reevaluated regarding its selectivity. Freshly isolated resting basophils expressed the highest level of IL-4 compared with other cell types. Because the basophil purification procedure requires more isolation steps, ex vivo manipulation might activate the cells. However, it should also be noted that basophils have been recently found to be the major source of IL-4, at least in asthma models.[37,38]

We thank Ms Noriko Hashimoto, Mr Keisuke Yuki, Mr Hisashi Tomita, Mr Nobuyuki Baba, and Ms Nao Aida at The National Research Institute for Child Health and Development for discussion and skillful technical assistance. We also thank Mr Igor A. Bagayev at The Confocal Microscope Facility, Massachusetts Genral Hospital, Harvard Medical School.

REFERENCES

1. Bochner BS. Systemic activation of basophils and eosinophils: markers and consequences. J Allergy Clin Immunol 2000;106(suppl):S292-302.
2. Hirai K, Miyamasu M, Takaishi T, Morita Y. Regulation of the function of eosinophils and basophils. Crit Rev Immunol 1997;17:325-52.
3. Savage CO. The evolving pathogenesis of systemic vasculitis. Clin Med 2002;2:458-64.
4. Oehler R, Weingartmann G, Manhart N, Salzer U, Meissner M, Schlegel W, et al. Polytrauma induces increased expression of pyruvate kinase in neutrophils. Blood 2000;95:1086-92.
5. Church MK, Pao GJ, Holgate ST. Characterization of histamine secretion from mechanically dispersed human lung mast cells: effects of anti-IgE, calcium ionophore A23187, compound 48/80, and basic polypeptides. J Immunol 1982;129:2116-21.
6. Lin CS, Boltz RC, Blake JT, Nguyen M, Talento A, Fischer PA, et al. Voltage-gated potassium channels regulate calcium-dependent pathways involved in human T lymphocyte activation. J Exp Med 1993;177:637-45.
7. Saito H, Okajima F, Molski TF, Sha'afi RI, Ui M, Ishizaka T. Effects of ADP-ribosylation of GTP-binding protein by pertussis toxin on immunoglobulin E-dependent and -independent histamine release from mast cells and basophils. J Immunol 1987;138:3927-34.
8. Ji TH, Grossmann M, Ji I. G protein-coupled receptors. I. Diversity of receptor-ligand interactions. J Biol Chem 1998;273:17299-302.
9. Zambrowicz BP, Sands AT. Knockouts model the 100 best-selling drugs. Will they model the next 100? Nat Rev Drug Discov 2003;2:38-51.
10. Venter JC, Adams MD, Myers EW, Li PW, Mural RJ, Sutton GG, et al. The sequence of the human genome. Science 2001;291:1304-51.
11. Waterston RH, Lindblad-Toh K, Birney E, Rogers J, Abril JF, Agarwal P, et al. Initial sequencing and analysis of the human genome. Nature 2001;409:860-921.
12. Drews J. Drug discovery: a historical perspective. Science 2000;287:1960-4.
13. Hopkins AL, Groom CR. The druggable genome. Nat Rev Drug Discov 2002;1:727-30.
14. Iida M, Matsumoto K, Tomita H, Nakajima T, Akasawa A, Ohtani NY, et al. Selective down-regulation of high-affinity IgE receptor (FcεRI) α-chain messenger RNA among transcriptome in cord blood-derived versus adult peripheral blood-derived cultured human mast cells. Blood 2001;97:1016-22.
15. Nakajima T, Matsumoto K, Suto H, Tanaka K, Ebisawa M, Tomita H, et al. Gene expression screening of human mast cells and eosinophils using high-density oligonucleotide probe arrays: abundant expression of major basic protein in mast cells. Blood 2001;98:1127-34.
16. Nakajima T, Inagaki N, Tanaka H, Tanaka A, Yoshikawa M, Tamari M, et al. Marked increase in CC chemokine gene expression in both human and mouse mast cell transcriptomes following Fc* receptor I cross-linking: an interspecies comparison. Blood 2002;100:3861-8.
17. Yoshimura C, Miyamasu M, Nagase H, Iikura M, Yamaguchi M, Kawanami O, et al. Glucocorticoids induce basophil apoptosis. J Allergy Clin Immunol 2001;108:215-20.
18. Matsumoto K, Schleimer RP, Saito H, Iikura Y, Bochner BS. Induction of apoptosis in human eosinophils by anti-Fas antibody treatment in vitro. Blood 1995;86:1437-43.
19. Okayama Y, Hunt TC, Kassel O, Ashman LK, Church MK. Assessment of the anti-c-kit monoclonal antibody YB5.B8 in affinity magnetic enrichment of human lung mast cells. J Immunol Methods 1994;169:153-61.
20. Fukagawa K, Okada N, Fujishima H, Nakajima T, Tsubota K, Takano Y, et al. CC-chemokine receptor 3: a possible target in treatment of allergy-related corneal ulcer. Invest Ophthalmol Vis Sci 2002;43:58-62.
21. Arock M, Schneider E, Boissan M, Tricottet V, Dy M. Differentiation of human basophils: an overview of recent advances and pending questions. J Leukoc Biol 2002;71:557-64.
22. Kepley CL, McFeeley PJ, Oliver JM, Lipscomb MF. Immunohistochemical detection of human basophils in postmortem cases of fatal asthma. Am J Respir Crit Care Med 2001;164:1053-8.

23. Loitto V-M, Forslund T, Sundqvist T, Magnusson K-E, Gustafsson M. Neutrophil leukocyte motility requires directed water influx. J Leukoc Biol 2002;71:212-22.
24. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001;29:e45.
25. Adra CN, Lelias JM, Kobayashi H, Kaghad M, Morrison P, Rowley JD, et al. Cloning of the cDNA for a hematopoietic cell-specific protein related to CD20 and the beta subunit of the high-affinity IgE receptor: evidence for a family of proteins with four membrane-spanning regions. Proc Natl Acad Sci U S A 1994;91:10178-82.
26. Adra CN, Mao XQ, Kawada H, Gao PS, Korzycka B, Donate JL, et al. Chromosome 11q13 and atopic asthma. Clin Genet 1999;55:431-7.
27. Donato JL, Ko J, Kutok JL, Cheng T, Shirakawa T, Mao XQ, et al. Human HTm4 is a hematopoietic cell cycle regulator. J Clin Invest 2002;109:51-8.
28. Nutku E, Aizawa H, Hudson SA, Bochner BS. Ligation of Siglec-8: a selective mechanism for induction of human eosinophil apoptosis. Blood 2003;101:5014-20.
29. O'Reilly M, Alpert R, Jenkinson S, Gladue RP, Foo S, Trim S, et al. Identification of a histamine H4 receptor on human eosinophils—role in eosinophil chemotaxis. J Recept Signal Transduct Res 2002;22:431-48.
30. Hirai H, Tanaka K, Yoshie O, Ogawa K, Kenmotsu K, Takamori Y, et al. Prostaglandin D2 selectively induces chemotaxis in T helper type 2 cells, eosinophils, and basophils via seven-transmembrane receptor CRTH2. J Exp Med 2001;193:255-61.
31. Moffatt JD, Jeffrey KL, Cocks TM. Protease-activated receptor-2 activating peptide SLIGRL inhibits bacterial lipopolysaccharide-induced recruitment of polymorphonuclear leukocytes into the airways of mice. Am J Respir Cell Mol Biol 2002;26:680-4.
32. Schmidlin F, Amadesi S, Vidil R, Trevisani M, Martinet N, Caughey G, et al. Expression and function of proteinase-activated receptor 2 in human bronchial smooth muscle. Am J Respir Crit Care Med 2001;164:1276-81.
33. Bradding P, Okayama Y, Kambe N, Saito H. Ion channel gene expression in human lung, skin and cord blood-derived mast cells. J Leukoc Biol 2003;73:614-20.
34. Gounni AS, Lamkhioued B, Koussih L, Ra C, Renzi PM, Hamid Q. Human neutrophils express the high-affinity receptor for immunoglobulin E (Fc epsilon RI): role in asthma. FASEB J 2001;15:940-9.
35. Koshino T, Teshima S, Fukushima N, Takaishi T, Hirai K, Miyamoto Y, et al. Identification of basophils by immunohistochemistry in the airways of post-mortem cases of fatal asthma. Clin Exp Allergy 1993;23:919-25.
36. Kepley CL, McFeeley PJ, Oliver JM, Lipscomb MF. Immunohistochemical detection of human basophils in postmortem cases of fatal asthma. Am J Respir Crit Care Med 2001;164:1053-8.
37. Schroeder JT, Lichtenstein LM, Roche EM, Xiao H, Liu MC. IL-4 production by human basophils found in the lung following segmental allergen challenge. J Allergy Clin Immunol 2001;107:265-71.
38. Luccioli S, Brody DT, Hasan S, Keane-Myers A, Prussin C, Metcalfe DD. IgE(+), Kit(-), I-A/I-E(-) myeloid cells are the initial source of Il-4 after antigen challenge in a mouse model of allergic pulmonary inflammation. J Allergy Clin Immunol 2002;110:117-24.

Genes and Immunity (2004) 5, 58–62
© 2004 Nature Publishing Group All rights reserved 1466-4879/04 $25.00
www.nature.com/gene

An asthma-associated genetic variant of STAT6 predicts low burden of ascaris worm infestation

G Peisong[1,6], X-Q Mao[2], T Enomoto[3], Z Feng[4], F Gloria-Bottini[5], E Bottini[5], T Shirakawa[2], D Sun[4] and JM Hopkin[1]

[1]*Experimental Medicine Unit, The Clinical School, University of Wales Swansea, UK;* [2]*Department of Health Promotion, Kyoto University Medical School, Kyoto, Japan;* [3]*Department of Allergy and Otolaryngology, Red Cross Hospital, Wakayama, Japan;* [4]*The Institute of Parasitic Disease, Shanghai, People's Republic of China;* [5]*Divisions of Medical Statistics and Preventive and Social Paediatrics, University of Rome Tor Vergata School of Medicine, Rome, Italy*

Th-2 immune mechanisms are involved in the pathology of asthma and in the protective immune response to parasitic worms. Common upregulating genetic variants of Th-2 immune signalling are risk factors for asthma, and we tested whether they may confer a counteradvantage in protecting against parasitic worms. We examined the intensity of infection by the parasitic worm, Ascaris lumbricoides, by microsopic counting of ascaris eggs in the stool of 614 schoolchildren from an area of endemic ascaris infection in China. We investigated the relationship between the intensity of ascaris infection and common, asthma-associated genetic variants of Th-2 and Th-1 immune signalling. Ascaris egg counts per gram of stool (epg), mean 1068 epg, ranged from barely detectable (<240 epg) to heavy (~9600 epg) in a skewed distribution. Logistic regression, after exploratory discriminant analysis, showed a major association between a common genetic variant of the 3′-UTR regulatory elements of the signal transducer and transactivating factor (STAT6) (P=0.0002) and egg counts, at the 77th centile. Linear regression after log transformation of egg counts confirmed a highly significant association with this STAT6 variant (P=0.001). Thus, a common, asthma-associated, genetic variant of the pivotal transduction and transactivating factor for Th-2 immune signalling, STAT6, predicts increased resistance to ascaris worm infection. The evolution of enhanced resistance to parasitic worm infection, through human genetic variation in Th-2 immune signalling, may represent one origin for asthma.
Genes and Immunity (2004) **5**, 58–62. doi:10.1038/sj.gene.6364030

Keywords: *STAT6; polymorphism; ascaris*

Introduction

Infestation with parasitic helminths is a natural condition for billions of humans in regions of the world where poor sanitation promotes rounds of infection and reinfection; children are notably susceptible to heavy infection when ill-health and poor growth result.[1] It has been repeatedly observed that there is a significant heterogeneity in the burden of helminth infestation in areas of endemic infection, in which only some 20% of any young population show a high intensity of infection and therefore carry most of the worms.[1,2] Environmental, behavioural and genetic factors are considered to contribute to this heterogeneity—but the detailed nature of the genetic factors in particular is not known.[3,4]

Asthma has become a highly prevalent chronic disease of children and young adults in developed countries.[5] Environmental factors, including changing patterns of early childhood infection and diet, are considered candidate mechanisms for the rapid rise of asthma,[6–8] but genetic factors are also important in the disease. The heterogeneity of asthma's genetic background is emphasised by the many chromosomal linkage results in genome-wide studies.[9]

It is notable that the bronchial pathology in asthma and the protective host response to helminth include prominent elements of Th-2 immune activity, which lead to increased mucosal eosinophil activity, mucus hypersecretion and muscle hyperactivity.[10,11] In the bronchus, these features underlie the chronic, episodic symptomatology of asthma.[10] In the gut, these immune mechanisms promote the expulsion of helminths.[11] Observational studies in humans, and knockout experiments in mice, emphasise the key roles of IL-13[12–14] and STAT6[11,15–17] in mediating these mucosal changes.

We, and others have found that common genetic variants of Th-2 immune signalling are significant predictors of asthma-including variants of IL-13,[18,19] IL-13Rα1,[18] IL-4Rα1[19,20] (which with IL-13Rα1 constitutes the IL-13 receptor) and STAT6.[21,22] Biological, functional and molecular modelling studies point to upregulation of Th-2 immune signalling in two of the variants tested, those in IL-13 and IL-4Rα1.[18,20,23]

We reasoned that the high prevalence of such variants, which promote asthma in early life, might derive from a counterbalancing evolutionary advantage of the variants in promoting protective Th-2 immune responses against high burdens of helminth infection. We therefore tested

Correspondence: JM Hopkin, Experimental Medicine Unit, The Clinical School, University of Wales Swansea. Swansea SA2 8PP, UK.
E-mail: j.m.hopkin@swansea.ac.uk
[6]Current address: Johns Hopkins Asthma and Allergic Centre, Baltimore, MD, USA. E-mail: pgao1@jhu.edu.
Received 23 April 2003; revised 28 July 2003; accepted 20 August 2003

whether these common asthma-promoting genetic variants might limit helminth burden in an area of China, where infection with the helminth *Ascaris lumbricoides* is endemic.

Methods

Study population

In total, 614 schoolchildren (319 males, 295 females), aged 11–15 years (mean 12.42), from four schools in the Xing Chang district of Shanghai were the subjects of the study. In the Xing Chang district, the majority of the population is involved in subsistence agriculture, many farmers still use fresh night soil to fertilise green-leaf vegetables, and ascaris infection is endemic.

Ascaris burden

Ascaris is a roundworm, in which there is faecal–oral spread of infection and reinfection, in approximately 1 billion people worldwide. Its lifecycle involves transient passage of blood-borne larvae across the lung, and the mature worms reside in the small intestine for their life span of 1 year, where the female worm excretes some 0.25 million eggs per day into the human faeces.[1] The number of eggs in the faeces is generally regarded as a reliable indicator of the burden of adult worm carriage in the intestine,[4,24] and high worm burdens lead to poor health and growth in children.[1]

The children we studied had stool samples examined by the Kato–Katz method[25] for the presence and numbers of ascaris eggs per gram of faeces (epg), in preparation for eradicative treatment. In addition, it was recorded whether the eggs of any other parasitic worms were identified, including hookworm species, trichuris and enterobius. Fresh stool was drawn through a 100-mesh gauze into plastic microscopy wells (with volumes defining the weight of stool), and helminth eggs were identified and counted by microscopy and expressed for each child as ascaris epg.

Questionnaire on social factors and health status

Each child responded to a questionnaire documenting their area of residence in the district, whether their house was traditional wood-built or modern brick-built, and whether their diet was based on foods of local origin or more mixed. In addition, the children responded to questions from a modified ISAAC questionnaire on symptoms of asthma, hay fever and eczema. The children were weighed, as an indicator of nutrition. The diameter of indurated skin response to tuberculin (2 tuberculin units, TU) was measured, as a marker of Th-1 immune response, and as a potential confounder of the helminth burden—genetic variant analysis.

Genetic analysis

Genetic analysis was conducted on DNA extracted from circulating white cells. Assays were performed for common genetic variants belonging to the Th-2 and Th-1 immune cytokine signalling pathway, which we had previously found to be associated with asthma in a Japanese or British population.[20,21,23,24] The loci and variants tested, the number of successful genotypes completed, the allele frequencies and heterozygosities are shown in Table 1. The genotype distributions at all loci tested matched Hardy–Weinberg equilibrium. All were single-nucleotide polymorphisms (SNPs) and were assayed by the Taqman TM high-throughput SNP screening method.[25] Fluorescent signal intensity was optimised by testing an array of primer concentrations using standard assay conditions. The sub-set of concentration pairs with two primers was derived empirically to reduce the number of reactions that had to be run for each assay optimisation. PCR products, amplified in triplicate for each set of concentrations, were analysed in an ABI prism 7700 sequence detector.

Statistical analysis

We tested for relationships between ascaris egg burden and (a) the genetic variant genotypes and (b) the social and health status variables recorded by questionnaire. The distribution of ascaris burden (Table 2) showed a continuous skewed distribution, and we employed a set of different statistical methods and utilised the Statistical Package for Social Sciences (SPSS).

Egg counts were log transformed and a linear regression analysis was performed, directly followed by stepwise procedures.

Exploratory discriminant analysis was used to test for genetic associations at specific levels of egg burden: (a) at

Table 1 Variant loci tested against ascaris burden in the Chinese schoolchildren

Locus	*Number of genotypes completed*	*Variant*	*Heterozygosity*	*Allele frequencies*
IL-4	606	Promoter (+33 C/T)	0.29	0.163, 0.837
IL-5	599	Promoter (−703 T/C)	0.31	0.294, 0.706
IL-13	605	Promoter (−1055 C/T)	0.39	0.196, 0.804
IL-13	579	Gln110Arg	0.36	0.332, 0.668
IL-4Rα1	602	Ile50Val	0.53	0.457, 0.543
IL-13Rα1	608	3′-UTR (1499 G/A)	0.27	0.471, 0.529
STAT6	608	3′-UTR (4219 G/A)	0.45	0.490, 0.510
IL-5Rα	606	Promoter (−80 G/A)	0.28	0.205, 0.795
FcεRIα	604	Promoter (−66 T/C)	0.07	0.037, 0.963
FcεRIβ	578	Intron 2 (1798 G/A)	0.27	0.161, 0.839
IL-10	608	Promoter (−627 C/A)	0.45	0.348, 0.652
IL-10R	602	Intron 1 (520 G/A)	0.49	0.365, 0.635
IFNγ	590	Intron 1 (1384 A/T)	0.26	0.139, 0.861
IL-12β2	594	3′-UTR (1159 G/A)	0.49	0.421, 0.579
IL-12Rβ2	593	Promoter (−325 G/T)	0.18	0.112, 0.888

Table 2 Frequency of different ascaris egg counts (epg) in a total of 614 subjects

Egg counts	*Number of individuals**	*Percentage*
<240	305	50.0
240–720	162	26.6
960–1680	86	14.3
1920–3360	32	5.2
3600–6000	12	2.0
6480–9600	13	2.1

*Four cases were unclassified.

the 77th centile (⩾960 epg), and matching the general recognition that 20% of most populations carry the highest worm burdens;[1,2] (b) the 50th centile (⩾240 epg, generally recognised in the Shanghai Health Programme as positive cases requiring eradicative treatment); (c) the 90th centile (⩾1920 epg) as a test at the very highest burdens. It was planned that any significant genetic effect within the discriminant analysis should be further investigated by logistic regression, employing direct and then step-wise procedures.

Results

Ascaris infection was common. The numbers of ascaris eggs in stool (geometric mean, 1068 epg) ranged greatly across the children, and the distribution matched the expectation that approximately 20% would show high burdens of worm infestation (Table 2). The geometric mean egg count (1068 epg) matches that in a recent study in Nepal[24] (1453 epg) and indicates a moderate worm burden. No hookworm or enterobius eggs were identified. In all, 99 of the children with ascaris infection also showed sparse numbers of trichuris eggs.

Linear regression analysis, after log transformation of the egg counts, identified a significant genetic association. After stepwise procedures, these genetic associations were attributable to the common genetic variants tested in STAT6 (P=0.001) and IL-4 (P=0.008).

The exploratory discriminant analysis showed significant genetic associations with egg burden at the 77th centile of egg counts (X^2=52.549, 24 degrees of freedom matching the number of genetic and environmental variables tested; P=0.001), but no significant association at either the 50th or 90th centiles. No associations were found between egg count at this centile with age, gender, body weight, tuberculin status, or any other behavioural or environmental variable. Table 3 shows the coefficients of discriminant analysis after a stepwise procedure, indicating that the variants of IFN-g, STAT6 and Il-13 were the principal associating factors. The subsequent stepwise logistic regression analysis at this 77th centile of egg count (Table 4) supported and amplified these indications; the genetic associations with egg burden were attributable principally to the variant of STAT6—operating independently (χ^2=13.89, P=0.0002), and operating through an interaction with the variant of IFN-γ (χ^2=7.65, P=0.006). A variant of IL-13 ligand contributed a lesser, but significant contribution (χ^2=4.02, P=0.045).

Table 3 Associations of genetic variants with ascaris egg count (⩾960) at the 77th centile

Genetic variants	*Coefficients of discriminant function after stepwise procedure*
IL-13	−0.41972
STAT 6	−0.50814
FcεR2	+0.21755
IL-12β	+0.21881
IFN-γ	−0.67165

The five most significant standardized canonical discriminant function coefficients (after the stepwise procedure of the discriminant analysis) are shown: the greatest coefficient values (positive or negative) were for IFN-g, STAT6, and Il-13.

Table 4 Associations of genetic variants with ascaris egg count at the 77th centile: logistic regression analysis, stepwise procedure

Variants	χ^2	*P-value*
STAT6	13.89	0.0002
Interaction (STAT6-IFNγ)	7.65	0.0060
IL-13	4.02	0.0450
Constant	34.77	0.0000

Homozygous GG (*vs* AG and AA) at 4219 STAT6 predicted protection against high, ascaris egg counts (⩾960 epg) with odds ratio 0.34, and 95% confidence intervals of 0.17–0.69. There was a significant trend (P=0.004) for decreasing mean egg counts according to genotype at 4219-AA (1417.22), AG (1195.09), GG (505.98). Figure 1 shows the trends for the STAT6 genotypes, by ascaris egg count.

Regular symptoms of asthma affected only seven of the 614 Chinese children studied, and therefore no analysis for associations with any of the genetic variable was attempted.

Discussion

The results of both linear regression and logistic regression at the 77th centile of egg counts indicate a strong association between allele G at nucleotide 4219 of STAT6, particularly in the homozygous state, with diminished ascaris burden in children living in an area of endemic infection. In our published study of asthma in an urban Japanese population, homozygous GG-4219 of STAT6 predicted chronic, regular treatment-requiring asthma in young adults with mild allergy at an odds ratio of 3.19, 95% confidence intervals 1.40–7.08.[21]

The data suggest that the G-4219 variant within the 3′ regulatory elements of STAT6, or another variant in tight linkage disequilibrium, may potentiate STAT6 activity and hence Th-2 immune effects, in turn promoting asthma in urban environments (free of ascaris infection), but protecting against ascaris infection in environments where there is endemic infection. This possibility is consistent with the central role of STAT6 in mediating Th-2 cytokine-induced eosinophil, mucus and muscle activity in both asthma[26] and in helminth infections.[11]

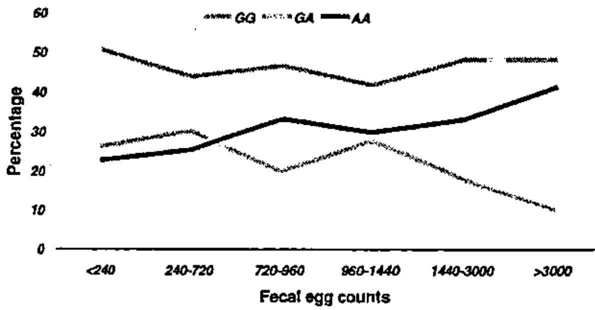

Figure 1 Plots of STAT6 variant genotypes—percentage prevalence at different faecal ascaris egg counts among 614 Chinese children.

Our data also indicate the action of other genetic variants of immune signalling in relation to ascaris burden—a variant of IL-4 in the linear regression, a variant of IFN-γ in an interaction with STAT6, and a variant of IL-13 operating at the 77th centile of burden in the logistic regression (Table 4). A polygenic interaction, with a major effect from the pivotal Th-2 transduction molecule (STAT6), in limiting worm burden also accords with the strong evolutionary pressure that must be exerted by endemic helminth infection in billions of humans, and the helminths' many evasive and subversive tactics, directed at diverse elements of the human's protective responses to the parasite.[1,27] The chromosomal location of STAT-6 (chromosome 12) does not match chromosomal linkages recently observed between ascaris burden and chromosomes 1 and 13 in a Nepalese genetic isolate (Jirels).[28] Genetic differences between the Chinese and Jirels may explain this discrepancy, but we have no information on STAT6 variation in the Jirels. With respect to our own discriminant and logistic regression analysis, it is notable that the relationship we observe between the STAT6 variant and ascaris burden in the Chinese population operates close to the well-recognised boundary of infection intensity for many helminths; in this ~20% of a human population is heavily infected and carry most of the worms, and are also most likely to incur reduced biological fitness due to ill-health and poor growth.[2]

Our data, based on one population, are essentially hypothesis generating. Further epidemiological studies are needed on genetic variants of STAT6, and variants of other Th2/Th1 signalling components, in relation to ascaris burden in other geographical areas of endemic infection and also in relation to the varying burden of other parasitic worm infections.

It is of interest that the 4219 variant of STAT6 lies in a C-rich region of the 3'UTR regulatory elements, since there is increasing documentation that these elements are major modulators of protein expression, through diverse actions including the determination of mRNA transcript half-life.[29,30] No natural coding sequence variants of STAT6 have been described—and no data are available on the functional impact of its noncoding variants and how these might underlie the association with asthmatic[21] and atopic phenotypes,[22,31] and now helminth burden. It is plausible that their actions should be on mRNA or protein quantity, and hence impact on the degree of STAT6-mediated transcriptional activation of many genes encoding the effector mechanisms of IL-4 and Il-13 signalling in Th-2 cells and at mucosae. Coding sequence variants of STAT6 have been induced by mutagenesis, and have been shown to influence the structure and stability of monomeric and dimeric STAT6 protein, and enhance STAT6 activity.[32] The epidemiological interaction of a variant of IFN-γ (the major cytokine of Th-1 immunity) with STAT6 in limiting worm burden raises different functional possibilities; that the IFN-γ variant may either up- or downregulate Th-1 activity, resulting in Th-2/Th1 cooperation or diminished antagonism by Th-1 of Th-2 mechanisms in limiting worm burden, or that high Th-1 function and low Th-2 activity promote high worm burdens. Detailed functional studies are now needed to clarify these various points.

Our data, recording the association between a common genetic variant of STAT6 with more asthma but less ascaris infection, may be relevant to the long-studied and complex relationship between helminth infections and asthma and allergy. Recent studies have recorded both inverse[33,34] and positive relationships[35] between helminth infection and asthma, but have not taken into account pertinent molecular genetic variants. Our data suggest that one element may be an inverse relationship, through genetic variation in Th-2 immune signalling, in which those with heavy worm burdens are least likely to manifest asthma. Other relationships must also exist, as suggested by recent studies in which helminth infections may inhibit allergy and asthma, through the induction of immune controlling factors, for instance the enhanced secretion of IL-10.[36] Future epidemiological studies into the relationship between worm infection and asthma/allergy therefore need to take into account genetic variants in Th-2 signalling.

Our findings suggest that one mechanism for asthma may be humans' evolutionary adaptation to ubiquitous helminth infection—operating in this instance through enhanced STAT6 and Th-2 activity. Further studies are now needed.

Acknowledgement

Dr Gao Peisong was supported by a doctoral studentship from Glaxo-Smith-Kline.

References

1 Maizels RM, Bundy DA, Selkirt ME, Smith DF, Anderson RM. Immunological modulation and evasion by helminth parasites in human populations. *Nature* 1993; 365: 797–805.
2 Woolhouse ME, Dye C, Etard JF *et al*. Heterogeneities in the transmission of infectious agents: implications for the design of control programs. *Proc Natl Acad Sci USA* 1997; 94: 338–342.
3 Zinn-Justin A, Marquet S, Hillaire D, Dessein A, Abel L. Genome search for additional human loci controlling infection levels by *Schistosoma mansoni*. *Am J Trop Med Hyg* 2001; 65: 754–758.
4 Williams-Blangero S, Subedi J, Upadhayay RP *et al*. Genetic analysis of susceptibility to infection with *Ascaris lumbricoides*. *Am J Trop Med Hyg*. 1999; 60: 921–926.
5 The International Study of Asthma and Allergies in Childhood (ISAAC) Steering Committee. Worldwide variation in prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and atopic eczema; ISAAC. *Lancet* 1998; 351: 1225–1232.
6 Shirakawa T, Enomoto T, Shimazu S, Hopkin JM. The inverse association between tuberculin responses and atopic disorder. *Science* 1997; 275: 77–79.

7 Martinez FD, Holt PG. Role of microbial burden in aetiology of allergy and asthma. *The Lancet* 1999; **354**: SII12–SII15.
8 Kankaanpaa P, Nurmela K, Erkkila A *et al.* Polyunsaturated fatty acids in maternal diet, breast milk, and serum lipid fatty acids of infants in relation to atopy. *Allergy* 2001; **56**: 633–638.
9 Xu J, Meyers DA, Ober C *et al.* Genome-wide screen and identification of gene–gene interactions for asthma-susceptibility loci in three US populations: collaborative study on genetics of asthma. *Am J Hum Genet* 2001; **68**: 1437–1446.
10 Lee JH, Kaminski N, Dolganov G *et al.* Interleukin-13 induces dramatically different transcriptional programs in three human airway cell types. *Am J Respir Cell Mol Biol* 2001; **25**: 474–485.
11 Urban Jr JF, Noben-Trauth N, Donaldson DD *et al.* IL-13, IL-4Rα and STAT6 are required for the expulsion of the gastrointestinal nematode parasite *Nippostrongylus brasiliensis*. *Immunity* 1998; **8**: 255–264.
12 Grunig G, Warnock M, Wakil AE *et al.* Requirement for IL-13 independently of IL-4 in experimental asthma. *Science* 1998; **282**: 2261–2263.
13 Walter DM, McIntire JJ, Berry G *et al.* Critical role for IL-13 in the development of allergen-induced airway hyperreactivity. *J Immunol* 2001; **167**: 4668–4675.
14 Finkelman FD, Wynn TA, Donaldson DD, Urgan JF. The role of IL-13 in helminth-induced inflammation and protective immunity against nematode infections. *Curr Opin Immunol* 1999; **11**: 420–426.
15 Urban Jr JF, Schopf L, Morris SC *et al.* STAT6 signalling promotes protective immunity against *Trichinella spiralis* through a mast cell- and T-cell-dependent mechanism. *J Immunol* 2000; **164**: 2046–2052.
16 Khan WI, Vallance BA, Blennerhassett PA *et al.* Critical role for signal transducer and activator of transcription factor 6 in mediating intestinal muscle hypercontractility and worm expulsion in *Trichinella spiralis*-infected mice. *Infect Immun* 2001; **69**: 838–844.
17 Khan WI, Blennerhasset P, Ma C, Matthaei KI, Collins SM. STAT6 dependent goblet cell hyperplasia during intestinal nematode infection. *Parasite Immunol* 2002; **23**: 39–42.
18 Heinzmann A, Mao XQ, Akaiwa M *et al.* Genetic variants of IL-13 signalling and human asthma and atopy. *Hum Mol Genet* 2000; **9**: 549–559.
19 Howard TD, Koppelman GH, Xu J *et al.* Gene–gene interaction in asthma: IL-4Rα and IL-13 in a Dutch population with asthma. *Am J Hum Genet* 2002; **70**: 230–236.
20 Mitsuyasu H, Izuhara K, Mao XQ *et al.* Ile50Val variant of IL-4Rα upregulates IgE synthesis and associates with atopic asthma. *Nat Genet* 1998; **19**: 119–120.
21 Gao PS, Mao XQ, Roberts MH *et al.* Variants of STAT6 (signal transducer and activator of transcription 6) in atopic asthma. *J Med Genet* 2000; **37**: 380–382.
22 Tamura K, Arakawa H, Suzuki M *et al.* Novel dinucleotide repeat polymorphism in the first exon of the STAT6 gene is associated with allergic diseases. *Clin Exp Allergy* 2001; **31**: 1509–1514.
23 Arima K, Umeshita-Suyama R, Sakata Y *et al.* Upregulation of IL-13 concentration *in vivo* by the IL13 variant associated with bronchial asthma. *J Allergy Clin Immunol* 2002; **109**: 980–987.
24 Forrester JE, Scott ME. Measurement of *Ascaris lumbricoides* infection intensity and dynamics of expulsion following treatment with mebendazole. *Parasitology* 1999; **100**: 303–308.
25 Gao PS. *Epidemiology of asthma and atopy: environmental and genetic studies* PhD Thesis, University of Wales, Swansea, 2002.
26 Mullings RE, Wilson SJ, Puddicome SM *et al.* Signal transducer and activator of transcription 6 (STAT6) expression and function in asthmatic bronchial epithelium. *J Allergy Clin Immunol* 2001; **108**: 832–838.
27 Culley FJ, Brown A, Girod N, Pritchard DI, Williams TJ. Eotaxin is specifically cleaved by hookworm metalloproteases preventing its action *in vitro* and *in vivo*. *J Immunology* 2000; **99**: 5533–5539.
28 Williams-Blangero S, Verde-Burg JL, Sabedi J *et al.* Genes on chromosome 1 and 13 have significant effects on ascaris infection. *Proc Natl Acad Sci* 2002; **99**: 5533–5538.
29 Conne B, Stutz A, Vassali JD. The 3′ untranslated region of messenger RNA: a molecular hot spot for pathology. *Nat Med* 2000; **6**: 637–641.
30 Wickens M, Anderson P, Jackson RJ. Life and death in cytoplasm: messages from the 3′ end. *Curr Opin Genet Dev* 1997; **7**: 220–232.
31 Amoli MM, Hand S, Hajeer AH *et al.* Polymorphism in the STAT6 gene encodes risk for nut allergy. *Genes Immun* 2002; **3**: 220–224.
32 Daniel C, Salvekar A, Schindler U. A gain-of-function mutation in STAT6. *J Biol Chem* 2000; **275**: 14255–14259.
33 van den Biggelaar AH, van Ree R, Rodrigues LC *et al.* Decreased atopy in children infected with *Schistosoma haematobium*: a role for parasite-induced interleukin-10. *Lancet* 2000; **356**: 1723–1727.
34 Scrivener S, Yemaneberhan H, Zebenigus M *et al.* Independent effects of intestinal parasite infection and domestic allergen exposure on risk of wheeze in Ethiopia: a nested case–control study. *Lancet* 2001; **358**: 1493–1499.
35 Palmer LJ, Celedon JC, Weiss ST, Wang B, Fang Z, Xu X. *Ascaris lumbricoides* infection is associated with increased risk of childhood asthma and atopy in rural China. *Am J Respir Crit Care Med* 2002; **165**: 1489–1493.
36 Yazdanbakhsh M, van den Biggelaar A, Maizels RM. Th-2 responses without atopy: immunoregulation in chronic helminth infections and reduced allergic disease. *Trends Immunol* 2001; **22**: 372–377.

INTERNATIONAL ARTICLE

Allergic Symptoms and Microflora in Schoolchildren

SANAE FUKUDA, Ph.D., HIROKI ISHIKAWA, Ph.D., YASUHIRO KOGA, M.D., Ph.D., YUJI AIBA, KAZUKO NAKASHIMA, M.M.Sc., LEI CHENG, M.D., AND TARO SHIRAKAWA, M.D., Ph.D.

We studied 867 junior high school children and administered a questionnaire documenting allergic symptoms and environmental variables, and measured Immunoglobulin E serum levels and the immunoglobulin G titers of serum antibody to microflora. A total of 716 subjects were ultimately used for statistics; those with at least two of the following allergic symptoms: asthma, rhinitis, eczema, or food allergy, showed significantly higher IgG titers to *Bactroides vulgatus* than other groups. This finding suggests that a species of the *Bacteroides* genus of the intestinal microflora tends to affect the gut issues, but further studies are needed to clarify this. © *Society for Adolescent Medicine, 2004*

KEY WORDS:
Allergy
Schoolchildren
Microflora

The prevalence of allergic diseases has been increasing in developed countries. However, many studies have demonstrated that the prevalence of these diseases is lower in the formerly socialist countries of Central and Eastern Europe than in Western European counties [1]. One factor associated with the rise of allergic diseases may be the decline of many infectious diseases in developed countries as the result of improved living standards and vaccinations [2]. In modern societies, it has been noted that allergies may be "mapped" according to gradients dictated by hygiene and by the individual degree of genetic predisposition to allergy [3]. These concepts are known as the "Hygiene hypothesis" [3–5]. It has been suggested that a reduced microbial stimulation during infancy and early childhood would result in slower postnatal maturation of the immune system and development of an optimal balance between Th1- and Th2-like immunities [6].

To clarify what and when bacterial species of commensal intestinal microbes are recognized as antigens that induce serum antibody responses in allergy patients, we studied the serum antibody responses to microflora in atopic school-aged children.

Methods

From a population of approximately 1000 12–13-year-old schoolchildren attending the 18 junior high schools in the country in 1995, we did a retrospective study of records of tuberculin responses in 867 children. They had been immunized with bacillus Calmette-Guerin (BCG) at 6 and 12 years of age and they showed negative tuberculin responses. Shirakawa et al showed the strong inverse association between delayed hypersensitivity to tuberculin and atopy [7]. The Institutional Review Board of RIKEN approved this study and 787 of all recruited subjects gave informed consent. We administered a questionnaire documenting atopic symptoms; the

From the Department of Health Promotion and Human Behavior, Kyoto University Graduate School of Public Health, Kyoto, Japan (S.F., K.N., L.C., T.S.); and Department of Infectious Diseases, School of Medicine, Tokai University, Isehara, Japan (H.I., Y.K., Y.A.).

Address correspondence to: Dr. Sanae Fukuda, Department of Health Promotion and Human Behavior, Kyoto University Graduate School of Public Health, Yoshida-Konoe, Kyoto 606-8501, Japan. E-mail: fukuda@pbh.med.kyoto-u.ac.jp

Manuscript accepted September 25, 2003.

1054-139X/04/$–see front matter
doi:10.1016/j.jadohealth.2003.09.018

© Society for Adolescent Medicine, 2004
Published by Elsevier Inc., 360 Park Avenue South, New York, NY 10010

Table 1. The Relationship Between Allergy Symptoms and IgG Titers of Serum Antibody Microflora in School Children (Mean ± SE)

Microflora or IgE	Nonallergy (n = 433)	One allergy symptom (n = 200)	2 or more allergy symptoms (n = 83)	F	p
E. coli	3.04 ± 0.01	3.07 ± 0.02	3.04 ± 0.03	0.62	.54
B. vulgatus	2.94 ± 0.01	2.91 ± 0.01	2.97 ± 0.02	3.00	.05
E. faecalis	2.99 ± 0.01	2.99 ± 0.02	3.03 ± 0.03	1.11	.33
B. longum	3.02 ± 0.01	3.01 ± 0.02	3.06 ± 0.02	1.38	.25
IgE	2.19 ± 0.03	2.25 ± 0.04	2.41 ± 0.08	4.23	.02

All were analyzed by one-way analysis of variance. Each gut flora or IgE values were converted to $\log_{10}$ because they didn't show normal distribution. SE = means standard error; *E. coli* = *Escherichia coli; B. vulgatus* = *Bacteroides vulgatus; E. faecalis* = *Enterococcus faecalis; B. longum* = *Bfidobacterium longum;* IgE = Immunoglobulin E.

questions were: "Do you have asthma?," "Do you have rhinitis?," "Do you have eczema?," "Do you have food allergy?" Also included were environmental variables, family allergy history, tuberculin response, etc., and we also measured immunoglobulin E (IgE) serum levels (measured by Mitsubishi BCL, Inc., Kyoto, Japan) and the immunoglobulin G (IgG) titers of serum antibody to *Escherichia coli* (*E. coli*), *Bacteroides vulgatus* (*B. vulgatus*), *Enterococcus faecalis* (*E. faecalis*), and *Bifidobacterium longun* (*B. longun*). This assay was described in detail in a previous article [8]. The titers of IgG class antibodies reacting with bacteria in serum were measured by enzyme-linked immunosorbent assay, and the absorbance was read at 495 nm using a plate reader.

We avoided subjects who did not complete questions about atopic symptoms and whose serum sample could not be measured for technical reasons. Ultimately we used 716 subjects for statistics. The values of the IgG titers of serum antibody to microflora and the level of IgE were converted to $\log_{10}$. Differences in IgG titers to *E. coli, B. vulgatus, E. faecalis*, and *B. longun* between nonallergic schoolchildren with no allergic symptoms, and symptomatic schoolchildren with more than one allergic symptom among asthma, rhinitis, eczema, and food allergy, were analyzed by two-tailed Student's *t*-test. We also tested the relation in IgG titers by each symptom; asthma, rhinitis, eczema, and food allergy by two-tailed Student's *t*-test. Then we divided subjects into three groups; "nonallergy," who have no allergy symptoms; "one allergy," who have one allergic symptom among asthma, rhinitis, eczema, and food allergy; and "two or more allergy," who have at least two allergic symptoms among asthma, rhinitis, eczema, and food allergy. We examined the differences in IgG titers to *E. coli, B. vulgatus, E. faecalis*, and *B. longun* among the three groups by one-way analysis of variance. The statistical analysis for this study was done using SPSS 10.0J for Win.

Results

IgG titers to *E. coli, B. vulgatus, E. faecalis*, and *B. longun* showed no significant difference between nonallergic schoolchildren (n = 433) with no allergic symptoms, and symptomatic schoolchildren (n = 283) who have more than one allergic symptom among asthma, rhinitis, eczema, and food allergy. We also tested the relation by each symptom; asthma, rhinitis, eczema, and food allergy; there were, however, no differences between nonallergic children and children with one or more allergic symptoms. The subjects were divided into three groups; "nonallergy" (n = 433), who have no allergy symptoms; "one allergy" (n = 200), who have one allergic symptom among asthma, rhinitis, eczema, and food allergy; and "two or more allergy" (n = 83) who have at least two allergic symptoms among asthma, rhinitis, eczema, and food allergy. In the "two or more allergy" group, IgG titers to *B. vulgatus* were significantly higher than in the other two groups (Table 1) (F = 3.00 df = 2, p = .05). IgG titers to *E. coli, B. vulgatus*, and *E. faecalis* showed no significant difference among three groups. Also in the "two or more allergy" group, subjects showed the highest mean IgE levels (2.41 ± 0.08 vs. 2.19 ± 0.03, 2.25 ± 0.04; F = 4.23 df = 2, p = .02) among three groups (Table 1).

Discussion

The children with two or more of the allergic symptoms showed higher IgG titers to *B. vulgatus* than the groups of nonallergic or one-allergic-symptom schoolchildren. This finding suggests that a species of the *Bacteroides* genus of the intestinal microflora might be an organism that affects the gut issues and thus, induces inflammation accompanied by an elevation of serum antibodies to this bacterium [8], although the exact mechanism of this remains un-

clear. Some studies reported that there was a difference in the composition of intestinal microflora between allergic and nonallergic infants. They compared 1-year-old infants living in two countries with a low (Estonia) and a high (Sweden) prevalence of atopy, and found that *Lactobacilli* and *Eubacteria* were more frequently found in the intestinal microflora of Estonian infants, whereas *Clostridia* was more prevalent in Swedish infants [9]; 2-year-old allergic children were colonized less often by *Lactobacilli*, and harbored higher counts of aerobic bacteria (coliforms, *Staphylococcus aureus*) than nonallergic children [10]. These subjects were, however, all infants, and the number of samples was small. Kirjavainen et al recently showed that, in 4.2–10.5-year-old children, serum total IgE concentration correlated with bacteroides in heightened risk of subjects with atopic disorders, and those subjects also showed a greater number of lactobacilli/enterococci than those in the low-risk group [11]. Our data is partly consistent with this.

This is the first study to clarify the relationship between atopic symptoms and bacterial species of predominant commensal intestinal microflora in a large group of junior high school children. Further studies are needed to clarify when bacterial species of commensal microbes in the gut are recognized as the antigens that induce serum antibody responses in allergic subjects.

References

1. Bjorksten B, Dumitrascu D, Foucard T, et al. Prevalence of childhood asthma, rhinitis and eczema in Scandinavia and Eastern Europe. Eur Respir J 1998;12:432–7.
2. Doll R. Health and the environment in the 1990s. Am J Public Health 1992;82:933–41.
3. Matricardi PM, Bonini S. High microbial turnover rate preventing atopy: A solution to inconsistencies impinging on the Hygiene hypothesis? Clin Exp Allergy 2000;30:1506–10.
4. Strachan DP. Hay fever, hygiene and household size. BMJ 1989;299:1259–60.
5. Martinez FD, Holt PG. Role of microbial burden in aetiology of allergy and asthma. Lancet 1999;354(Suppl 2):SII12–5.
6. Bottcher MF, Nordin EK, Sandin A, et al. Microflora-associated characteristics in faeces from allergic and nonallergic infants. Clin Exp Allergy 2000;30:1590–6.
7. Shirakawa T, Enomoto T, Shimazu S, Hopkin JM. The inverse association between tuberculin responses and atopic disorder. Science 1997;275:77–9.
8. Saitoh S, Noda S, Aiba Y, et al. Bacteroides ovatus as the predominant commensal intestinal microve causing a systemic antibody response in inflammatory bowel disease. Clin Diagn Lab Immunol 2002;9:54–9.
9. Sepp E, Julge K, Vasar M, et al. Intestinal microflora of Estonian and Swedish infants. Acta Pediatr 1997;86:956–61.
10. Bjorksten B, Naaber P, Sepp E, Mikelsaar M. The intestinal microflora in allergic Estonian and Swedish 2-year-old children. Clin Exp Allergy 1999;29:342–6.
11. Kirjavainen PV, Arvola T, Salminen SJ, Isolauri E. Aberrant composition of gut microbiota of allergic infants: a target of bifidobacterial therapy at weaning? Gut 2002;51:51–5.

Hum Genet (2004) 114 : 503–509
DOI 10.1007/s00439-004-1099-5

ORIGINAL INVESTIGATION

Mitsuteru Akahoshi · Mami Ishihara · Natascha Remus
Kazuko Uno · Katsuhisa Miyake · Tomomitsu Hirota
Kazuko Nakashima · Akira Matsuda · Mizuo Kanda
Tadao Enomoto · Shigeaki Ohno · Hitoshi Nakashima
Jean-Laurent Casanova · Julian M. Hopkin
Mayumi Tamari · Xiao-Quan Mao · Taro Shirakawa

Association between *IFNA* genotype and the risk of sarcoidosis

Received: 24 September 2003 / Accepted: 3 February 2004 / Published online: 5 March 2004
© Springer-Verlag 2004

Abstract Sarcoidosis is known to be a systemic granulomatous disorder characterized by a cell-mediated Th1-type inflammatory response. To identify a key genetic factor in the pathogenesis of sarcoidosis, we investigated single nucleotide polymorphisms within 10 candidate genes involved in type 1 immune process (*IFNA17, IFNB, IFNG, IFNGR1, IFNGR2, IL12B, IL12RB1, IL12RB2, ETA-1*, and *NRAMP1*) in an association-based study of 102 Japanese patients with sarcoidosis, 114 with tuberculosis, and 110 control subjects. After correction for multiple testing, an *IFNA17* polymorphism (551T→G) was found to be associated with susceptibility to sarcoidosis (odds ratio 3.27 [95% CI: 1.44–7.46], P=0.004, P_c=0.04), but not to tuberculosis. We observed no significant associations with the other polymorphisms of the Th1-related genes. We further typed another *IFNA* polymorphism (*IFNA10* 60T→A) and confirmed two major haplotypes of the *IFNA* gene, viz., allele 1: *IFNA10* [60T]-*IFNA17* [551T] and allele 2: *IFNA10* [60A]-*IFNA17* [551G], in the Japanese population. In healthy subjects, *IFNA* allele 2, which is over-represented in patients with sarcoidosis, was significantly associated with increased IFN-α and IL-12p70 production induced by Sendai virus in vitro. This study suggests that possession of the *IFNA* allele with higher levels of IFN-α significantly increases the risk of sarcoidosis.

M. Akahoshi and M. Ishihara contributed equally to this work

M. Akahoshi · T. Hirota · A. Matsuda · M. Tamari · T. Shirakawa
Laboratory for Genetics of Allergic Diseases,
SNP Research Center,
Institute of Physical and Chemical Research,
RIKEN Yokohama Institute, Yokohama, Japan

M. Ishihara
Department of Ophthalmology,
Yokohama City University School of Medicine, Yokohama, Japan

N. Remus · J.-L. Casanova
Laboratory for Human Genetics of Infectious Diseases,
INSERM U550, Necker Medical School,
University of Paris Rene Descartes, Paris, France

K. Uno
Louis Pasteur Center for Medical Research, Kyoto, Japan

M. Akahoshi · K. Miyake · H. Nakashima
Department of Medicine and Biosystemic Science,
Graduate School of Medical Sciences, Kyushu University,
Fukuoka, Japan

M. Kanda
Kanda Hospital, Wakayama, Japan

T. Enomoto
Department of Otolaryngology, Wakayama Medical Center,
Japanese Red Cross Society, Wakayama, Japan

A. Matsuda · S. Ohno
Department of Ophthalmology,
Hokkaido University School of Medicine, Sapporo, Japan

J. M. Hopkin
Experimental Medicine Unit, University of Wales at Swansea,
Swansea, UK

K. Nakashima · X.-Q. Mao · T. Shirakawa (✉)
Department of Health Promotion and Human Behavior,
Graduate School of Public Health, Kyoto University,
Yoshida-konoe, 606–8501 Kyoto, Japan
Tel.: +81-75-7534451, Fax: +81-75-7534452,
e-mail: shirakawa@pbh.med.kyoto-u.ac.jp

Introduction

Sarcoidosis is a chronic systemic disorder that has an unknown etiology and that affects a wide range of organs. Its pathological hallmark is the noncaseating granuloma and it is further characterized by the accumulation and activation of T lymphocytes and macrophages at sites of ongoing inflammation, notably in the lung. Studies of cytokines and cellular mechanisms in sarcoidosis suggest that T cells in affected organs are of the T helper 1 (Th1) phenotype, producing interferon-γ (IFN-γ) and interleukin-2 (IL-2; ATS/ERS/WASOG Committee 1999; Newman et al. 1997). Thus, a cell-mediated Th1-type immune response appears to play a major role in the disease pathogenesis.

Although the cause of sarcoidosis remains obscure, time-, space-, and work-related clustering suggests that the disease shares some common environmental triggers

(ATS/ERS/WASOG Committee 1999; Newman et al. 1997). Several transmissible agents or infectious organisms including viruses, *Propionibacterium acnes*, and mycobacterium, have been implicated as possible agents (ATS/ERS/WASOG Committee 1999), but definitive evidence and the identification of such agents are still lacking. In addition, there are differences in the pattern of organ involvement and the severity of the disease. US blacks, for instance, are more acutely and severely affected than other races (ATS/ERS/WASOG Committee 1999). Thus, racial and ethnic variations in the disease prevalence and familial clustering suggest a genetic contribution to the etiology. Currently, the general concept regarding its pathogenesis is that it results from the exposure of genetically susceptible individuals to specific environmental agents.

Mycobacterium tuberculosis is another granulomatous lung disease associated with the development of cell-mediated immunity. Studies of patients with selective susceptibility to poorly pathogenic mycobacterial and *Salmonella* infections have revealed genetic mutations in the type 1 cytokine pathway (Casanova and Abel 2002). Therefore, common genetic polymorphisms related to the Th1 cascade might contribute to susceptibility to intracellular infectious diseases such as tuberculosis.

Results of genetic studies have shown that sarcoidosis is a complex heritable disorder with many factors that alter the disease phenotype, and to date, several candidate genes, such as *ACE*, *CCR2*, *IL1*, and *CR1*, have been proposed to contribute to the susceptibility to sarcoidosis (Iannuzzi et al. 2002). Recently, gene polymorphisms of vascular endothelial growth factor and Clara cell 10-kDa protein have also been reported as susceptibility genes in Japanese patients with sarcoidosis (Morohashi et al. 2003; Ohchi et al. 2004). To our knowledge, however, few studies have examined the functional consequences of these variants. We hypothesize that a particular gene encoding a molecule involved in the Th1-mediated immune process is a candidate gene for susceptibility to sarcoidosis. Our aims have been to examine associations between previously confirmed polymorphisms in Th1-related genes and the development of sarcoidosis in Japanese, to compare their results with tuberculosis cases, and to investigate the functional relevance of disease-related polymorphisms.

Materials and methods

Patients and controls

We enrolled 102 Japanese patients with sarcoidosis (mean age: 36.2 years; range: 12–69 years; 78 women, 24 men); they were recruited mainly from the Department of Ophthalmology at the Yokohama City University School of Medicine and Japanese Red Cross Medical Center, between 1989 and 1993. Sarcoidosis was diagnosed on the basis of the radiographic and clinical presentation, including uveitis, and the finding of noncaseating granulomas in biopsy specimens with negative special stains and cultures for acid-fast bacilli and fungus. Controls were 115 healthy sex-matched Japanese blood volunteers with an age range of 20–60 years who had no history of pulmonary disease or other inflammatory disease. For disease controls, we also enrolled 114 patients with smear-positive pulmonary tuberculosis (mean age: 53.3 years, SD=14.5; 17 women, 97 men) from Kanda Hospital in Wakayama. All subjects were unrelated Japanese living in central Japan. Informed consent was obtained from all the subjects prior to the study, and project approval was granted by the ethics committee at the SNP Research Center, Institute of Physical and Chemical Research (RIKEN).

Table 1 Candidate genes, polymorphisms, restriction enzymes, and PCR primers

Gene	Polymorphism	Restriction enzyme	Primers Forward primer 5' Reverse primer 5'
IFNA17	551 T/G	*Ssp*I	CAATCAGGATCATTGCCATG GCTTTGGACTTCCCCAGG
IFNB	153 C/T	*Cac8*I	CTGAAAGGGAGAAGTGAAAGTGG GTGCTAGATGAATCTTGTCTG
IFNG	1348 T/A	*Bgl*II	CTTACAACACAAAATCAGATC TTCCTGTAGGGTATTATT
IFNGR1	167 T/C	*Cac8*I	CGGGGTTGGAGCCAGCGAC CCTCCCTCCCTCTCGT
IFNGR2	839 G/A	*Hin*fI	CAGCTGCCCGCTCCTCAG GGCTTACTATTTAAACTGGACT
IL12B	1146 C/A	*Taq*I	TGGAGGAAAAGTGGAAGA GTATGATGGCACTGGTATCAG
IL12RB1	641 A/G	*Pvu*II	AGTCCTGGACAATTCTTACG GCTGGAACTACAGGTGGTGTGCA
IL12RB2	365 C/T	*Nco*I	GACATCTGCGAGGAAAGTTC AGTTCAGCTCTGGAAGCG
ETA-1 (SPP1)	2514 C/T	*Alu*I	ATATTCCCATCCCTAGCCGT GACTAAATGCAAAGTGAGAAATTG
NRAMP1 (SLC11A1)	1703 G/A	*Ava*II	GCATCTCCCCAATTCATGGT AACTGTCCCACTCTATCCTG