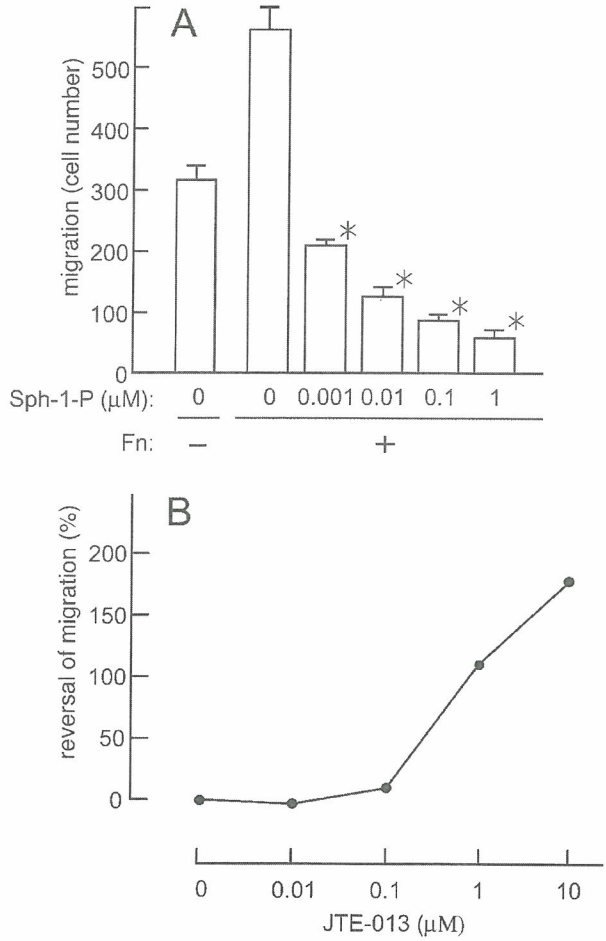

Fig. 3. **Inhibition of fibronectin-induced RBL-2H3 cell migration by Sph-1-P and its reversal by JTE-013.** (A) Various concentrations of Sph-1-P were placed in the lower chamber with (+) or without (–) 100 μg/ml of fibronectin (Fn). RBL-2H3 cells were allowed to migrate for 4 h. *Statistically significant compared with the control fibronectin-treated cells (without Sph-1-P treatment). (B) RBL-2H3 cells preincubated with various concentrations of JTE-013 for 10 min were allowed to migrate for 4 h toward the lower chamber, where 100 nM Sph-1-P was present or absent, together with 100 μg/ml of fibronectin. The reversal by JTE-013 of the Sph-1-P-inhibited migration (%) was calculated as ([migrating cells in the presence of JTE-013/Sph-1-P] – [migrating cells in the presence of Sph-1-P]) / ([migrating cells in the absence of JTE-013/Sph-1-P] – [migrating cells in the presence of Sph-1-P]) × 100.

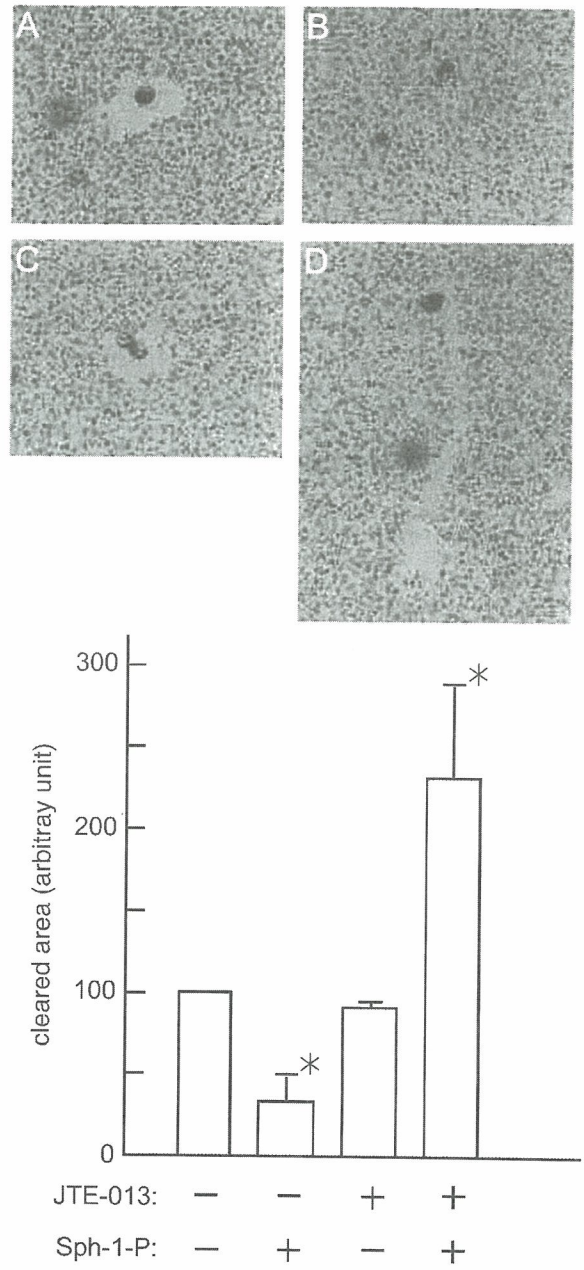

Fig. 4. **Inhibition of RBL-2H3 cell migration by Sph-1-P and its reversal by JTE-013, as assessed with a phagokinetic assay.** (Upper panel) RBL-2H3 cells were preincubated without (A and B) or with (C and D) 10 μM JTE-013 for 10 min, and then challenged without (A and C) or with (B and D) 1 μM Sph-1-P for 24 h. Chemokinesis was evaluated with a phagokinetic assay using gold sol-coated plates. (Lower panel) RBL-2H3 cells were treated as described for the upper panel, and the area cleared of gold particles for each cell was measured. *Statistically significant compared with the control cells (without Sph-1-P/JTE-013 treatment).

more, the fibronectin-stimulated cell migration in the presence of Sph-1-P plus JTE-013 was greater than that without Sph-1-P/JTE-013 (Fig. 3B).

Inhibition of RBL-2H3 Cell Phagokinesis by Sph-1-P and Its Reversal by a Specific $S1P_2$ Antagonist—To confirm the anti-motility effect of Sph-1-P, we also performed a phagokinetic track assay. The phagokinetic activity of RBL-2H3 cells on gold sol-coated plates was inhibited by Sph-1-P (Fig. 4). JTE-013, which by itself failed to affect the response, reversed the Sph-1-P-inhibited phagokinesis (Fig. 4). These results further strengthen the idea that Sph-1-P inhibits RBL-2H3 motility through $S1P_2$. Again, the response in the presence of Sph-1-P plus JTE-013 was greater than that without any treatment (Fig. 4).

Cytoskeletal Reorganization of RBL-2H3 Cells Treated with Sph-1-P—We further evaluated cytoskeletal reor-

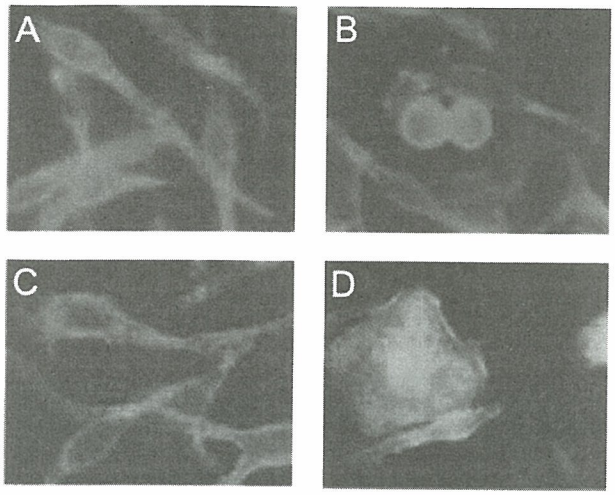

Fig. 5. **Cytoskeletal Reorganization of RBL-2H3 cells treated with JTE-013 and/or Sph-1-P.** RBL-2H3 cells were pretreated without (A and B) or with (C and D) 1 μM JTE-013 for 10 min, and then stimulated without (A and C) or with (B and D) 100 nM Sph-1-P for 30 min. Then, the cells were fixed, permeabilized, and stained with tetramethylrhodamine isothiocyanate-phalloidin for actin staining.

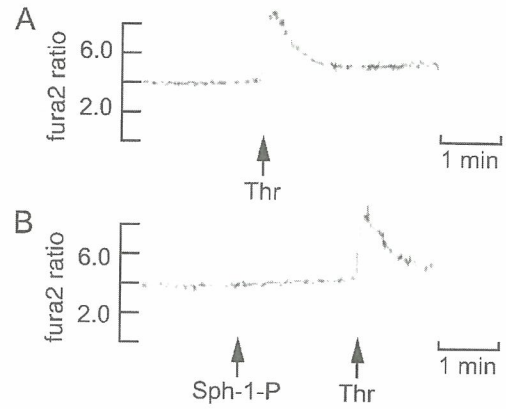

Fig. 6. **Measurement of RBL-2H3 cell $[Ca^{2+}]_i$ Fura2-loaded RBL-2H3 cells were stimulated with 1U/ml of thrombin (A) or 100 nM Sph-1-P and then 1U/ml of thrombin (B).** $[Ca^{2+}]_i$ was monitored as the ratio of fura2 fluorescence.

ganization under the conditions in which Sph-1-P exerted its anti-motility effect; RBL-2H3 cells stained to visualize F-actin are shown in Fig. 5. Low nanomolar concentrations of Sph-1-P induced stress fiber formation in RBL-2H3 cells (data not shown), possibly through Rho activation (*36*). When higher concentrations (100 nM – 1 μM) of Sph-1-P were employed, marked changes in cell morphology and increased numbers of rounded cells were observed (Fig. 5, A and B). The $S1P_2$ antagonist JTE-013, which, by itself, failed to affect the morphology of RBL-2H3 cells, blocked the Sph-1-P-induced cell shape change (Fig. 5), indicating Sph-1-P-induced cell rounding *via* $S1P_2$, as previously reported for transfected HEK293 cells (*37*). Instead, membrane ruffling-like structures were observed (Fig. 5D).

Effects of Sph-1-P on Intracellular Ca^{2+} Mobilization and Histamine Release in RBL-2H3 Cells—We next examined intracellular Ca^{2+} mobilization using Ca^{2+}-sensitive fluorophore fura2. As previously reported (*38*), thrombin caused a rapid and transient increase in $[Ca^{2+}]i$ (Fig. 5A). However, Sph-1-P, at concentrations capable of exerting the anti-motility effect, neither elicited an increase in $[Ca^{2+}]i$ nor affected the response induced by thrombin (Fig. 6B). When very high concentrations (above 10 μM) of Sph-1-P were employed, a small but significant increase was observed (data not shown), which should be independent of the effect of this bioactive lipid on RBL-2H3 cell migration shown above.

Consistent with the inability of Sph-1-P to induce intracellular Ca^{2+} mobilization (at least at nanomolar concentrations), this bioactive lysophospholipid failed to elicit histamine degranulation (data not shown), which is dependent on the Ca^{2+} signal (*39*, *40*).

Imitation by a Supernatant Prepared from Activated Platelets of the Effect Triggered by Sph-1-P—We finally analyzed platelet-mast cell interactions and the relative involvement of Sph-1-P in this cell-cell communication. For this purpose, we prepared a supernatant from an activated platelet suspension. This supernatant strongly inhibited RBL-2H3 cell migration (Table 2), as did Sph-1-P. Similar results were obtained when the boiled (instead of non-boiled) supernatant was used to eliminate the effect of peptide mediators (data not shown). Accordingly, not a protein but probably a lipid component seemed to be responsible for the migration inhibition by the platelet supernatant. Although a variety of bioactive substances are released from activated platelets, ones interacting with RBL-2H3 cells or mast cells have hardly been reported. Accordingly, we postulated that the observed effect of the supernatant may be due to the presence of Sph-1-P released from platelets, based on the resemblance of the effects of Sph-1-P and the platelet supernatant. We tested this possibility by examining the effect of the $S1P_2$ antagonist JTE-013 on the response induced by the supernatant; Sph-1-P inhibits RBL-2H3 migration through $S1P_2$, as shown above. As expected, the migration inhibition of RBL-2H3 cells by both the boiled (Fig. 7A) and non-boiled (Fig. 7B) activated platelet supernatants was completely reversed by JTE-013. Furthermore, pretreatment with high concentrations of this $S1P_2$ antagonist before the addition of the supernatant even enhanced the response compared with the control without any treatment (Fig. 7).

Table 2. **Inhibition of RBL-2H3 cell migration by a supernatant prepared from activated platelets.** RBL-2H3 cells were allowed to migrate for 4 h to the lower chamber, where various concentrations of a supernatant prepared from activated platelets were placed.

Experiment	Supernatant from activated platelets				
	0%	0.1%	1%	10%	100%
1	240	250	200	80	5
2	230	230	202	85	8
3	145			50	2
4	143			54	3

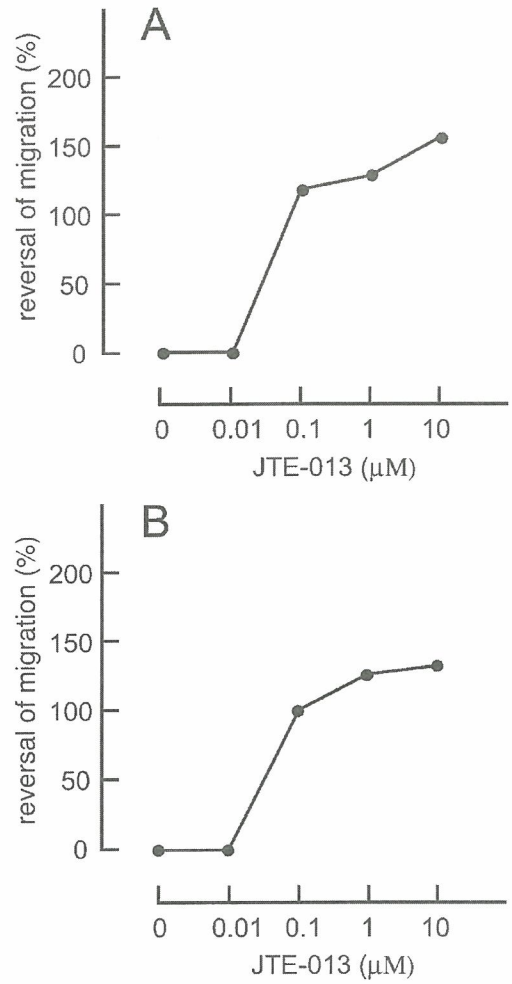

Fig. 7. **The reversal by JTE-013 of Sph-1-P-inhibited RBL-2H3 cell migration.** RBL-2H3 cells preincubated with various concentrations of JTE-013 for 10 min were allowed to migrate for 4 h toward the lower chamber, where a buffer containing 10% of the supernatant prepared from activated platelets was placed. The supernatant was boiled (B) or not boiled (A). The reversal by JTE-013 of the supernatant-inhibited migration (%) was calculated as ([migrating cells in the presence of JTE-013/the supernatant] – [migrating cells in the presence of the supernatant]) / ([migrating cells in the absence of JTE-013/the supernatant] – [migrating cells in the presence of the supernatant]) × 100.

DISCUSSION

Sph-1-P Inhibition of RBL-2H3 Cell Migration—In this study, we found that Sph-1-P strongly inhibits the migration of RBL-2H3 cells through the G protein-coupled receptor $S1P_2$. This is consistent with the facts that $S1P_2$ is strongly expressed in these cells (this study), and that $S1P_2$ negatively regulates membrane ruffling and the resultant cell migration (*22*, *34*). Although $S1P_2$ has been reported to be a chemorepellant receptor (*22*, *34*), our present results show that Sph-1-P interaction with $S1P_2$ leads to inhibition of both chemokinesis (random motility) and chemotaxis, since the phagokinetic activity of RBL-2H3 cells on gold sol-coated plates was inhibited by Sph-1-P, which was reversed by the $S1P_2$ antagonist JTE-013. Whether this difference is due to the choice of cell type remains to be solved.

It should be noted that, in cells pretreated with JTE-013 and then challenged with Sph-1-P, the migration and phagokinesis responses inhibited by Sph-1-P were not only blocked but also enhanced compared with those without Sph-1-P/JTE-013. RBL-2H3 cells express both $S1P_1$ and $S1P_2$. $S1P_1$ and $S1P_2$ exert contrasting effects on cell motility; the former stimulates membrane ruffling and migration in a Rac-dependent manner, while the latter inhibits these responses (*22*, *34*). Accordingly, when the $S1P_2$-mediated effect was blocked by its specific antagonist, the $S1P_1$-mediated effect, *i.e.*, Rac-dependent stimulation of migration, should be observed in Sph-1-P-challenged RBL-2H3 cells; Rac is involved in the migration of these cells (*41*). This is consistent with the morphological study in which a rounded cell shape (without cytosolic extension) was observed for RBL-2H3 cells incubated with Sph-1-P, while this rounded shape was abolished and membrane ruffling-like structures were observed when JTE-013 was pretreated before Sph-1-P addition.

Sph-1-P in RBL-2H3 Cells or Mast Cells: an Extracellular First Messenger or an Intracellular Second Messenger?—Sph-1-P is now considered to be a unique cell signaling molecule, functioning as both an extracellular first messenger and an intracellular second messenger (*20*). We believe that the regulation of RBL-2H3 cell motility by Sph-1-P reported in this study can be best explained by its extracellular action via the $S1P_2$ receptor since (i) $S1P_2$ is actually expressed in these cells, (ii) Sph-1-P is capable of eliciting the response at low nanomolar concentrations, corresponding to the Kd values of S1P receptors (*21*, *22*), and (iii) most importantly, the Sph-1-P-induced response was specifically blocked by an $S1P_2$ antagonist.

Our present paper is not the first to report the effect of exogenous Sph-1-P on mast cells or RBL-2H3 cells. Choi *et al.* reported that Sph-1-P increases intracellular Ca^{2+} in RBL-2H3 cells, although an extremely high concentration of Sph-1-P (such as 25 μM) was employed (*40*). It is hard to speculate that that high concentration of Sph-1-P can be attained *in vivo*; even the Sph-1-P concentration in serum, where the highest concentration of Sph-1-P could be attained given that the most important source of extracellular Sph-1-P is blood platelets, is less than 1 μM (*26*). In fact, it was postulated by Choi *et al.* that Sph-1-P, transiently formed intracellularly by sphingosine (Sph) kinase, acts as an intracellular messenger in signaling by the FcεRI antigen receptor (*40*). This Sph kinase-mediated Ca^{2+} signal, which is responsible for FcεRI-triggered mast cell degranulation, was later shown in human bone marrow-derived mast cells (*39*). Furthermore, high intracellular levels of Sph-1-P were shown to activate the mitogen-activated protein kinase pathway; again, as high as 10 μM Sph-1-P was employed in that study (*42*). Judging from the above together with our present study, it may be possible to speculate that both types of Sph-1-P action, *i.e.*, those as an intracellular messenger and an extracellular mediator, can be seen in mast cells; neither Ca^{2+} mobilization nor histamine release, possibly due to the intracellular Sph-1-P action, was observed with the

present conditions under which the anti-motility effect of extracellular Sph-1-P through $S1P_2$ was observed. This is not so surprising since a similar situation can be found in several systems (*18*, *20*). For example, in human umbilical vein endothelial cells, Sph-1-P induces migration, proliferation, angiogenesis, and nitric oxide formation through cell surface receptors $S1P_1$ and $S1P_3$ (*43*, *44*), while the Sph kinase pathway, through the generation of intracellular Sph-1-P, is critically involved in mediation of TNFα-induced endothelial activation (including adhesion molecule expression) (*45*).

Cross-talk between Mast Cells and Platelets—Another important finding in this study is that platelets seem to regulate mast (RBL-2H3) cell motility by releasing Sph-1-P. This is based on the facts that the Sph-1-P effect, *i.e.*, regulation of RBL-2H3 cell motility, is mimicked by a supernatant (both with and without boiling) prepared from activated platelets and that this effect of the supernatant was blocked by an $S1P_2$ antagonist. Given that 140 pmol Sph-1-P is stored in 10^8 platelets (*26*) and that 30% of it is released upon activation (*19*), the concentration of Sph-1-P in the supernatant prepared from activated platelets (5×10^8/ ml) is calculated to be 210 nM. Based on our results regarding the concentration-dependent effect of Sph-1-P (see Figs. 2A and 3A), this concentration of Sph-1-P is expected to exert an anti-motility effect on RBL-2H3 cells. At a site of inflammation, injury, or hemorrhage, platelets extravasate from the blood and adhere to the subendothelial tissue, which leads to their activation, while mast cells are residential cells adjacent to the endothelium in the connective and mucosal tissues. Therefore, activated platelets recruited to the inflamed or damaged site may modulate mast cell functions *in vivo*. The limitation of this study is the use of tumor mast cell line RBL-2H3, although this has been used frequently as an experimental model of mucosal mast cells (*27*). In this context, it should be noted that $S1P_1$ and $S1P_2$ are reportedly expressed not only in RBL-2H3 cells but also in primary bone marrow-derived mast cells (*25*). Furthermore, to strengthen our conclusion, other approaches than the usage of $S1P_2$ antagonists, *e.g.*, knockdown strategies involving siRNA, may be needed in the future.

We recently examined the effect of Sph-1-P on eosinophils. Sph-1-P acts as a chemoattractant for these cells (Yokoo, E., Yatomi, Y., Takafuta, T., and Ozaki, Y., unpublished observation), in contrast to mast cells. Accordingly, it is possible to speculate that Sph-1-P may inhibit and stimulate the locomotion of mast cells and eosinophils, respectively, thereby promoting interaction between mucosal mast cells (residing around the blood vessels) and infiltrating eosinophils. Recently, it was shown that nerve growth factor collaboratively worked with membrane lysophosphatidylserine of activated platelets to induce mast cell activation, and it was proposed that NGF released in response to inflammatory stimuli may contribute to mast cell activation in collaboration with locally activated platelets in the process of inflammation and tissue repair (*46*). We feel more attention should be paid to cross-talk between mast cells and platelets, although the significance of this cell-cell communication *in vivo* remains to be clarified in further studies.

The authors wish to thank the Japan Tobacco Incorporation and Prof. Takashi Morita for donating the JTE-013 and convulxin, respectively. This study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan, and by the Mitsubishi Pharma Research Foundation.

REFERENCES

1. Turner, H. and Kinet, J.P. (1999) Signalling through the high-affinity IgE receptor Fc epsilonRI. *Nature* **402**, B24–30
2. Nadler, M.J., Matthews, S.A., Turner, H., and Kinet, J.P. (2000) Signal transduction by the high-affinity immunoglobulin E receptor Fc epsilon RI: coupling form to function. *Adv. Immunol.* **76**, 325–355
3. Gresele, P., Ribaldi, E., Grasselli, S., Todisco, T., and Nenci, G.G. (1987) Evidence for platelet activation in allergic asthma. *Agents Actions* **21**, 119–128
4. Page, C.P. (1989) Platelets as inflammatory cells. *Immunopharmacology* **17**, 51–59
5. Averill, F.J., Hubbard, W.C., Proud, D., Gleich, G.J., and Liu, M.C. (1992) Platelet activation in the lung after antigen challenge in a model of allergic asthma. *Am. Rev. Respir. Dis.* **145**, 571–576
6. Mannaioni, P.F., Di Bello, M.G., and Masini, E. (1997) Platelets and inflammation: role of platelet-derived growth factor, adhesion molecules and histamine. *Inflamm. Res.* **46**, 4–18
7. Zucker-Franklin, D. and Philipp, C.S. (2000) Platelet production in the pulmonary capillary bed: new ultrastructural evidence for an old concept. *Amer. J. Pathol.* **157**, 69–74
8. Metzger, W.J., Sjoerdsma, K., Richerson, H.B., Moseley, P., Zavala, D., Monick, M., and Hunninghake, G.W. (1987) Platelets in bronchoalveolar lavage from asthmatic patients and allergic rabbits with allergen-induced late phase responses. *Agents Actions* **21**, 151–159
9. Beasley, R., Roche, W.R., Roberts, J.A., and Holgate, S.T. (1989) Cellular events in the bronchi in mild asthma and after bronchial provocation. *Am. Rev. Respir. Dis.* **139**, 806–817
10. Yamamoto, H., Nagata, M., Tabe, K., Kimura, I., Kiuchi, H., Sakamoto, Y., Yamamoto, K., and Dohi, Y. (1993) The evidence of platelet activation in bronchial asthma. *J. Allergy Clin. Immunol.* **91**, 79–87
11. Chihara, J., Yasuba, H., Tsuda, A., Urayama, O., Saito, N., Honda, K., Kayaba, H., Yamashita, T., Kurimoto, F., and Yamada, H. (1997) Elevation of the plasma level of RANTES during asthma attacks. *J. Allergy Clin. Immunol.* **100**, S52–55
12. Moritani, C., Ishioka, S., Haruta, Y., Kambe, M., and Yamakido, M. (1998) Activation of platelets in bronchial asthma. *Chest* **113**, 452–458
13. Hasegawa, S., Pawankar, R., Suzuki, K., Nakahata, T., Furukawa, S., Okumura, K., and Ra, C. (1999) Functional expression of the high affinity receptor for IgE (FcepsilonRI) in human platelets and its intracellular expression in human megakaryocytes. *Blood* **93**, 2543–2551
14. Kameyoshi, Y., Dorschner, A., Mallet, A.I., Christophers, E., and Schroder, J.M. (1992) Cytokine RANTES released by thrombin-stimulated platelets is a potent attractant for human eosinophils. *J. Exp. Med.* **176**, 587–592
15. Klouche, M., Klinger, M.H., Kuhnel, W., and Wilhelm, D. (1997) Endocytosis, storage, and release of IgE by human platelets: differences in patients with type I allergy and nonatopic subjects. *J. Allergy Clin. Immunol.* **100**, 235–241
16. Yoshimi, Y., Fujimura, M., Myou, S., Tachibana, H., and Hirose, T. (2001) Effect of thromboxane A2 (TXA2) synthase inhibitor and TXA2 receptor antagonist alone and in combination on antigen-induced bronchoconstriction in guinea pigs. *Prostaglandins Other Lipid Mediat.* **65**, 1–9
17. Dogne, J.M., de Leval, X., Benoit, P., Rolin, S., Pirotte, B., and Masereel, B. (2002) Therapeutic potential of thromboxane inhibitors in asthma. *Expert Opin. Investig. Drugs* **11**, 275–281

18. Yatomi, Y., Ozaki, Y., Ohmori, T., and Igarashi, Y. (2001) Sphingosine 1-phosphate: synthesis and release. *Prostaglandins Other Lipid Mediat.* **64**, 107–122
19. Yatomi, Y., Yamamura, S., Ruan, F., and Igarashi, Y. (1997) Sphingosine 1-phosphate induces platelet activation through an extracellular action and shares a platelet surface receptor with lysophosphatidic acid. *J. Biol. Chem.* **272**, 5291–5297
20. Spiegel, S. and Milstien, S. (2002) Sphingosine 1-phosphate, a key cell signaling molecule. *J. Biol. Chem.* **277**, 25851–25854
21. Kluk, M.J. and Hla, T. (2002) Signaling of sphingosine-1-phosphate *via* the S1P/EDG-family of G-protein-coupled receptors. *Biochim. Biophys. Acta* **1582**, 72–80
22. Takuwa, Y. (2002) Subtype-specific differential regulation of Rho family G proteins and cell migration by the Edg family sphingosine-1-phosphate receptors. *Biochim. Biophys. Acta* **1582**, 112–120
23. Ammit, A.J., Hastie, A.T., Edsall, L.C., Hoffman, R.K., Amrani, Y., Krymskaya, V.P., Kane, S.A., Peters, S.P., Penn, R.B., Spiegel, S., and Panettieri, R.A., Jr. (2001) Sphingosine 1-phosphate modulates human airway smooth muscle cell functions that promote inflammation and airway remodeling in asthma. *FASEB J.* **15**, 1212–1214
24. Racke, K., Hammermann, R., and Juergens, U.R. (2000) Potential role of EDG receptors and lysophospholipids as their endogenous ligands in the respiratory tract. *Pulm. Pharmacol. Ther.* **13**, 99–114
25. Jolly, P., Rosenfeldt, H., Milstien, S., and Spiegel, S. (2002) The roles of sphingosine-1-phosphate in asthma. *Mol. Immunol.* **38**, 1239–1245
26. Yatomi, Y., Igarashi, Y., Yang, L., Hisano, N., Qi, R., Asazuma, N., Satoh, K., Ozaki, Y., and Kume, S. (1997) Sphingosine 1-phosphate, a bioactive sphingolipid abundantly stored in platelets, is a normal constituent of human plasma and serum. *J. Biochem.* **121**, 969–973
27. Barsumian, E.L., Isersky, C., Petrino, M.G., and Siraganian, R.P. (1981) IgE-induced histamine release from rat basophilic leukemia cell lines: isolation of releasing and nonreleasing clones. *Eur. J. Immunol.* **11**, 317–323
28. Osada, M., Yatomi, Y., Ohmori, T., Ikeda, H., and Ozaki, Y. (2002) Enhancement of sphingosine 1-phosphate-induced migration of vascular endothelial cells and smooth muscle cells by an EDG-5 antagonist. *Biochem. Biophys. Res. Commun.* **299**, 483–487
29. Ikeda, H., Satoh, H., Yanase, M., Inoue, Y., Tomiya, T., Arai, M., Tejima, K., Nagashima, K., Maekawa, H., Yahagi, N., Yatomi, Y., Sakurada, S., Takuwa, Y., Ogata, I., Kimura, S., and Fujiwara, K. (2003) Antiproliferative property of sphingosine 1-phosphate in rat hepatocytes involves activation of Rho via Edg-5. *Gastroenterology* **124**, 459–469
30. Cicmil, M., Thomas, J.M., Sage, T., Barry, F.A., Leduc, M., Bon, C., and Gibbins, J.M. (2000) Collagen, convulxin, and thrombin stimulate aggregation-independent tyrosine phosphorylation of CD31 in platelets. Evidence for the involvement of Src family kinases. *J. Biol. Chem.* **275**, 27339–27347
31. Lynch, K.R. (2002) Lysophospholipid receptor nomenclature. *Biochim. Biophys. Acta* **1582**, 70–71
32. Graler, M.H., Bernhardt, G., and Lipp, M. (1998) EDG6, a novel G-protein-coupled receptor related to receptors for bioactive lysophospholipids, is specifically expressed in lymphoid tissue. *Genomics* **53**, 164–169
33. Im, D.S., Heise, C.E., Ancellin, N., O'Dowd, B.F., Shei, G.J., Heavens, R.P., Rigby, M.R., Hla, T., Mandala, S., McAllister, G., George, S.R., and Lynch, K.R. (2000) Characterization of a novel sphingosine 1-phosphate receptor, Edg-8. *J. Biol. Chem.* **275**, 14281–14286
34. Okamoto, H., Takuwa, N., Yokomizo, T., Sugimoto, N., Sakurada, S., Shigematsu, H., and Takuwa, Y. (2000) Inhibitory regulation of Rac activation, membrane ruffling, and cell migration by the G protein-coupled sphingosine-1-phosphate receptor EDG5 but not EDG1 or EDG3. *Mol. Cell. Biol.* **20**, 9247–9261
35. Suzuki, T., Shoji, S., Yamamoto, K., Nada, S., Okada, M., Yamamoto, T., and Honda, Z. (1998) Essential roles of Lyn in fibronectin-mediated filamentous actin assembly and cell motility in mast cells. *J. Immunol.* **161**, 3694–3701
36. Yamamura, S., Hakomori, S., Wada, A., and Igarashi, Y. (2000) Sphingosine-1-phosphate inhibits haptotactic motility by overproduction of focal adhesion sites in B16 melanoma cells through EDG-induced activation of Rho. *Ann. N. Y. Acad. Sci.* **905**, 301–307
37. Van Brocklyn, J.R., Tu, Z., Edsall, L.C., Schmidt, R.R., and Spiegel, S. (1999) Sphingosine 1-phosphate-induced cell rounding and neurite retraction are mediated by the G protein-coupled receptor H218. *J. Biol. Chem.* **274**, 4626–4632
38. Ali, H., Tomhave, E.D., Richardson, R.M., Haribabu, B., and Snyderman, R. (1996) Thrombin primes responsiveness of selective chemoattractant receptors at a site distal to G protein activation. *J. Biol. Chem.* **271**, 3200–3206
39. Melendez, A.J. and Khaw, A.K. (2002) Dichotomy of Ca^{2+} signals triggered by different phospholipid pathways in antigen stimulation of human mast cells. *J. Biol. Chem.* **277**, 17255–17262
40. Choi, O.H., Kim, J.-H., and Kinet, J.-P. (1996) Calcium mobilization via sphingosine kinase in signalling by the FceRI antigen receptor. *Nature* **380**, 634–636
41. Woo, C.H., Jeong, D.T., Yoon, S.B., Kim, K.S., Chung, I.Y., Saeki, T., and Kim, J.H. (2002) Eotaxin induces migration of RBL-2H3 mast cells *via* a Rac-ERK-dependent pathway. *Biochem. Biophys. Res. Commun.* **298**, 392–397
42. Prieschl, E.E., Csonga, R., Novotny, V., Kikuchi, G.E., and Baumruker, T. (1999) The balance between sphingosine and sphingosine-1-phosphate is decisive for mast cell activation after Fc epsilon receptor I triggering. *J. Exp. Med.* **190**, 1–8
43. Lee, M.J., Thangada, S., Claffey, K.P., Ancellin, N., Liu, C.H., Kluk, M., Volpi, M., Sha'afi, R.I., and Hla, T. (1999) Vascular endothelial cell adherens junction assembly and morphogenesis induced by sphingosine-1-phosphate. *Cell* **99**, 301–312
44. Lee, M.J., Thangada, S., Paik, J.H., Sapkota, G.P., Ancellin, N., Chae, S.S., Wu, M., Morales-Ruiz, M., Sessa, W.C., Alessi, D.R., and Hla, T. (2001) Akt-mediated phosphorylation of the G protein-coupled receptor EDG-1 is required for endothelial cell chemotaxis. *Mol. Cell* **8**, 693–704
45. Xia, P., Gamble, J.R., Rye, K.A., Wang, L., Hii, C.S., Cockerill, P., Khew-Goodall, Y., Bert, A.G., Barter, P.J., and Vadas, M.A. (1998) Tumor necrosis factor-a induces adhesion molecule expression through the sphingosine kinase pathway. *Proc. Natl Acad. Sci. USA* **95**, 14196–14201
46. Kawamoto, K., Aoki, J., Tanaka, A., Itakura, A., Hosono, H., Arai, H., Kiso, Y., and Matsuda, H. (2002) Nerve growth factor activates mast cells through the collaborative interaction with lysophosphatidylserine expressed on the membrane surface of activated platelets. *J. Immunol.* **168**, 6412–6419

Clin Exp All Rev 2004; 4:31–34

Prevalence of Japanese cedar pollinosis in children aged under 15 years throughout Japan

K. Okubo, M. Gotoh and M. Okuda
Department of Otolaryngology, Nippon Medical School, Tokyo, Japan

Summary
The prevalence of childhood allergic rhinitis is increasing steadily in Japan. Affected children are sensitized mainly by house dust mites; however, the prevalence of Japanese cedar pollinosis (JCP) in children is also on the rise. To ascertain the prevalence and current status of JCP in Japanese children, a retrospective analysis of a nationwide cross-sectional random sampling study was conducted. The survey, conducted shortly after the peak pollen season, was performed by self-evaluation questionnaire in 2001. Data from children aged under 15 years were collected and analysed. In these subjects, the response rate was 75.1%. The prevalence of JCP was 10.2% in children. Rhinorrhoea was the severest symptom reported; 19.5% had severe or moderate interference with daily activities and consulted physicians. Few children used prescribed drugs and some took measures to avoid contact with allergens.

Keywords children, epidemiology, Japanese cedar pollinosis, prevalence, allergic rhinitis

Introduction

Japanese cedar pollinosis (JCP) is a common allergic disease in Japan caused by inhalation of the pollen of Japanese cedar (*Cryptomeria japonica*) [1]. This disease is a major public health problem in Japan because of the severity of symptoms, high prevalence, poor spontaneous recovery rate and the cost of controlling the disease. Moreover, the prevalence in children is believed to have gradually increased in recent years [2]. According to a survey by Okuda et al. [3], the age-adjusted prevalence of JCP is 17.3% in the Japanese population as a whole, reduced to 13.1% after correction for possible biasing factors. However, although these data are useful, clearer understanding of the disease prevalence, variation in severity, limitations on activities of daily living and efficacy of current treatment and prevention strategies is needed for health-care policy planning and development of new treatment modalities and drugs in children with JCP.

Hence the present study was conducted to determine the current epidemiological prevalence of JCP in Japanese children using a cross-sectional random sampling method applied to the data from Okuda's survey.

Correspondence: Dr Kimihiro Okubo, Department of Otolaryngology, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113–8603, Japan.
E-mail: ent-kimi@nms.ac.jp

Method

Subjects

For the analysis, Japan was divided into 12 regional zones. Two-step stratified random sampling was performed in each zone. First, 390 of 3370 Japanese cities, towns and villages were selected randomly by the probability proportional sampling method in proportion to the overall population with respect to age (3–79 years) and sex on the basis of the National Census Report 1995. Following this, two subjects in each of the seven age/sex groups (14 subjects in total for each factor) were sampled randomly from the residents registration lists of the aforementioned 390 locations, and a list of 10 920 subjects was generated as reported previously [3].

Questionnaires

Self-evaluation questionnaires were mailed to all 10 920 subjects between 20 April and 2 May 2001. The questionnaire (see Appendix I) comprised 12 questions on symptoms (runny nose, sneezing, stuffy nose and eye itching) and their severity, changes after treatment (worse/improved/none), occurrence during each year, seasonal variability, frequency of JCP in the family, physician visits and clinical diagnosis, types of anti-JCP drugs used and their efficacy, degree by which JCP interferes with daily activities, methods used to avoid pollen and average duration of daily outdoor activities. Details of age and sex were also requested. Adult subjects were requested to

complete the questionnaires themselves; children were asked to respond with the assistance of a parent or guardian.

Diagnostic criteria

In the questionnaire-based diagnosis of JCP, definite JCP was diagnosed in subjects reporting ≥ 2 of a total of three nasal and one eye severe symptoms, with recurrence during February–April for more than 2 years or aggravation during this time if subjects reported perennial symptoms. Subjects whose symptoms had decreased to 'mild' or 'no symptoms' after treatment or occurred for the first time during 2001 were also deemed as having JCP. Suspected JCP was diagnosed in subjects who had ≥ 2 severe symptoms during the pollen season with occasional recurrence throughout the year as well as in those who had recurrence during the pollen season but whose symptoms were mild before treatment [3].

Results

Response rate

Fifty-four of the 10 920 questionnaires mailed out were returned to sender as the subjects were no longer at the addresses. Of the remaining 10 866 questionnaires, 5836 subjects (53.7%) responded. Of these returned questionnaires, 238 were discarded due to different age reporting from that given in the original mailed list or not specifying age or sex. Hence the usable response rate was 52.6% (5598 of 10 628), among which 1303 (23.3%) were children aged < 15 years. Of the usable responses from those questionnaires returned from children aged < 15 years, 655 were from male subjects and 648 from female subjects. Two hundred and two questionnaires were answered by the children themselves, 1090 by parents, and 11 unknown. The severity of each symptom in these responders is shown in Table 1.

Prevalence

The crude prevalence of JCP was estimated at 17.3% (966 of 5598) in the total population. Adjusting for the 53.7% response rate, the prevalence was presumed 12.2% (95% confidence interval: 7.6–16.8%) for a 100% response. The prevalence in those aged under 15 years was estimated at 10.2% (133 of 1303 responders). With regard to sex, the prevalence of JCP in male subjects (12.4%) was higher than in female subjects (8.0%) in that age group. The prevalence rates generally increased with age (Fig. 1). Stratified by age, the prevalence was 4.5%, 10.5%, 12.1% and 15.1% in those aged 3–5, 6–9, 10–12 and 13–15 years, respectively (Fig. 2).

Table 1. Positive rate of symptoms in 1303 children aged < 15 years throughout Japan

Symptom	No response	Severe	Mild	None
Sneezing, *n* (%)	24 (1.8)	105 (8.1)	290 (22.3)	884 (67.8)
Rhinorrhoea, *n* (%)	17 (1.3)	187 (14.1)	318 (24.4)	781 (59.9)
Nasal obstruction, *n* (%)	24 (1.8)	173 (13.3)	288 (22.1)	818 (62.8)
Eye itching, *n* (%)	30 (10.1)	131 (10.1)	240 (18.4)	902 (69.2)

Current status of JCP

The current status of JCP was analysed in 133 affected children who conformed to the study criteria (questionnaire-diagnosed JCP). Recurrence in each year was seen in 78.9% of the subjects, whereas 21.1% had JCP for the first time during the year of completion of the questionnaire. In terms of the pollen season, 51.9% of the subjects had symptoms only between February and April. In contrast, 48.1% had perennial symptoms with aggravation during the pollen season, suggesting that those with allergic rhinitis had symptoms precipitated by house dust mites or other allergens in association with JCP.

Of 133 children with questionnaire-diagnosed JCP, 66.9% had sought medical advice from physicians. Among these subjects, 62.4% were prescribed drugs from physicians and 17.0% took over-the-counter medications (first-generation antihistamines). These rates are higher than those observed in the total population [3]. With respect to the degree of JCP interference with daily activities, 23.3% reported severe interference, 27.8% moderate difficulty and 34.6% mild problems. Among subjects who received drug therapy, interference with daily activities was severe in only 5.5%, moderate in 18.2% and mild in 37.4%.

The most frequently used measures to evade pollen exposure were gargling (removal of pollen from throat; 48.1%), avoiding exposure of mattresses to sunshine (32.3%) and keeping windows and doors closed (25.6%). Wearing facemasks (18.0%) and eye washing (3.8%) were less common strategies for avoiding pollen in children.

Discussion

The prevalence of JCP in children aged under 15 years throughout Japan was investigated by a cross-sectional population analysis with use of random sampling. Recently, Baba reported the national prevalence of JCP at 16.2% in a household study of otolaryngologists conducted by mailed questionnaires [4]. However, this nonrandomized family study did not sample from the general population, unlike our randomized study. Baba's study found prevalence rates of 1.7% and 7.5% in 0–4- and 5–9-year-old children, respectively [4]. These rates are a little lower than those revealed by our random sampling method. The different contents of the respective questionnaires may have affected the results and led to the difference in estimation of prevalence.

Internationally, many questionnaires pertaining to allergic rhinitis have been made available, including those of

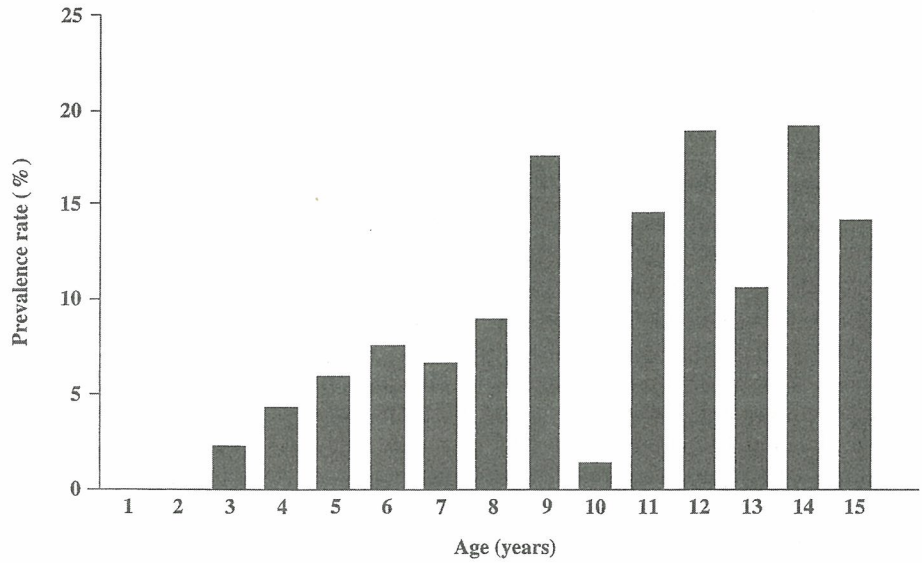

Fig. 1. Prevalence rate of JCP in children.

the International Survey of Asthma and Allergies in Childhood (ISAAC) formulated by the British Medical Research Council, the European Community Health Survey Questionnaire, the South London Survey, the American Thoracic Society questionnaire, and the Swiss Community Survey [5, 6]. According to these studies, allergic rhinitis prevalence rates in their respective populations were around 16–20%. However, none of these studies were conducted on a national basis. In Japan, surveys of JCP have been compiled by the Tokyo Metropolitan Government in addition to those conducted by Tanihara et al. [7], Sakakura and Ukai [8], Kozasa and Takenaka [2], and Okuda [9]. In general, all of these questionnaires had similar contents. However, the results varied considerably due to differences in sample size and year of study. The current study was conducted between April and July 2001, just after the peak pollen season when the subjects' perception of the severity of their symptoms was likely to be most accurate. During that period, the major airborne pollen in Japan was derived from the Japanese cedar tree, although small amounts of cypress pollen were also detected.

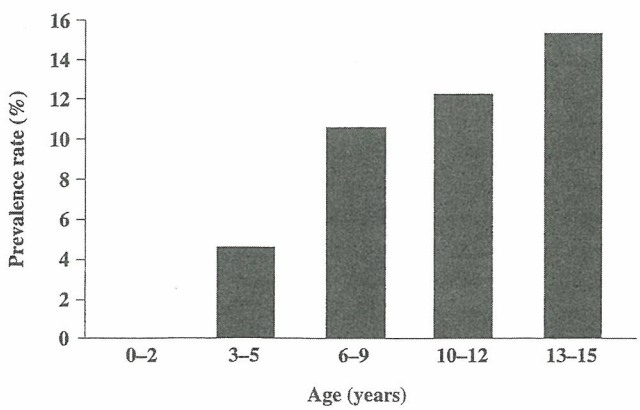

Fig. 2. Prevalence rate of JCP in children stratified by age.

The prevalence rates for JCP were low in the age groups comprising of subjects younger than 5 years old. No substantial differences in JCP prevalence were noted between the sexes in the total population, although male outnumbered female responders in the group aged < 15 years. This national survey revealed that patients had symptoms affecting both the nose and eyes. Nasal symptoms were more troublesome than eye symptoms in most children with JCP. The rate of medical treatment given was 18.2% of the total 5598 responders. On the other hand, 8.6% of 1303 responders aged < 15 years were treated by clinicians. Among those who received pharmacotherapy, the number of subjects experiencing severe interference in daily activities was less than the number of subjects experiencing mild or moderate interference in daily activities. This suggests that pharmaceutical intervention is effective in improving quality of life. Among those with questionnaire-diagnosed JCP aged < 15 years, the frequency of experiencing severe or moderate interference with daily activities before and after drug treatment was 51.1% and 23.7%, respectively. The relief of JCP symptoms is difficult, although patients may prevent symptoms altogether by avoiding the allergen. In the current study, gargling and keeping windows closed were often used as prophylactic measures. Although pollen masks have been shown to exclude approximately 60% of pollen inhaled from a pollen chamber [10], our children were not accustomed to seek prophylaxis by wearing masks and glasses.

Conclusion

The current study clearly demonstrates that JCP is a common disease in Japanese children and hence a public health problem because of its high prevalence (> 10% of Japanese children affected) and morbidity (causing severe or moderate interference with daily activity in 51.1% of patients).

Nevertheless, drug therapy and pollen avoidance seem very effective for symptomatic relief. Encouragingly, almost 66.9% of those affected visited physicians.

References

1 Okuda M, Shida T. Clinical aspects of Japanese cedar pollinosis. Allergol Int 1998; **47**:1–8.
2 Kozasa T, Takenaka H. Epidemiology of Japanese cedar pollinosis. Jibi Rinsyou 1995; **76** (Supplement):20–5 [in Japanese].
3 Okuda M. Epidemiology of Japanese cedar pollinosis throughout Japan. Ann Allergy Asthma Immunol 2003; **91**:288–96.
4 Baba K. Prevalence of Japanese cedar pollinosis. Prog Med 2000; **20**:2411–5 [in Japanese].
5 Variations in the prevalence of respiratory symptoms, self-reported asthma attacks, and use of asthma medication in the European Community Respiratory Health Survey (ECRHS). Eur Respir J 1996; **9**:687–95.
6 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**:1225–32.
7 Tanihara Oki I, Ojima T, Nakamura Y, Yanagihara H. Process and current status of the epidemiologic study on cedar pollinosis in Japan. J Epidemiol 1999; **29**:2–6.
8 Sakakura Y, Ukai K. Epidemiologic study of Japanese cedar pollinosis in Mie prefecture. In: Report of Research Project of Allergic Diseases. Tokyo, Japan: Ministry of Health and Welfare, 1995:211–4 [in Japanese].
9 Okuda M. Diagnostic criteria of Japanese cedar pollinosis in a population study. In: Report on Management and Prophylaxis of Pollinosis. Tokyo, Japan: Ministry of Health and Welfare, 1992:9–29 [in Japanese].
10 Xiao SF, Tanimoto H, Okuda M. Inhibitory effect of half-face masks on inhalation of particles of carbon powder and Japanese cedar pollen. Am J Rhinol 1991; **5**:57–60.

Appendix 1

Self-evaluation questionnaire for Japanese cedar pollinosis

Japanese cedar pollinosis is a common allergic disease in Japan caused by inhalation of Japanese cedar pollen. It is characterized by symptoms such as sneezing, runny nose, stuffy nose and eye itching, especially during the season February to April, and usually recurs annually. At present, the disease presents considerable health problems because of its high incidence rates and severe symptoms, but the numbers of people affected in the country as a whole are unknown. This national survey is designed to clarify the status of Japanese cedar pollinosis in Japan.

1 Please fill out the questionnaire and send it back to us as soon as possible.
2 Please note that we are asking you about the condition of your nose and eyes during February through April of this year when you did not have a cold or flu.
3 Please answer each question by checking the appropriate number in each item.

Q1a: Symptoms. Did/do you have the following symptoms during February through April of this year when you did not have a cold or flu? (Please check the severity of each symptom.)
(See Table 1 for list of symptoms and severity)

Q1b: Treatment effect. (Please answer only when you did not have any severe nose or eye symptoms.)

Did you have any severe symptoms before treatment that changed to mild or no symptoms after treatment?

1 Yes
2 No
3 Other

(Please proceed to Q8 if you had/have no symptom and checked 'no' for all symptoms.)

Q2: Recurrence. Do your nose/eye symptoms occur repeatedly almost every year?

1 Almost every year
2 Manifested for the first time this year
3 Some years, not every year
4 Other

Q3: Season. What is/was the most frequent season for your symptom(s) to appear?

1 February to April alone
2 Perennial, but worsens in February to April
3 Worse in another season or perennial
4 Other

Q4a: Consultation with a physician. Did you visit a physician this year for your symptoms?

1 Yes
2 No

Q4b: Physician's diagnosis. What was the physician's diagnosis?

1 Japanese cedar pollinosis
2 Pollinosis due to a plant other than cedar
3 Dust mite rhinitis
4 Other allergic rhinitis
5 Non-allergic rhinitis
6 Other/undefined

(Q5 through Q10 are not included in this Appendix because they are not crucial for the diagnosis of Japanese cedar pollinosis.)

Clin Exp All Rev 2004; 4:21–25

Allergic rhinitis in children: association with asthma

N. Shimojo*, S. Suzuki*, M. Tomiita*, Y. Inoue*, K. Nakano† and Y. Kohno*

**Department of Pediatrics, Graduate School of Medicine, Chiba University and †Department of Otolaryngology, Chiba Municipal Aoba Hospital, Chiba, Japan*

Summary

The incidence of allergic rhinitis in paediatric bronchial asthma patients was about 80% according to a questionnaire survey. Watery nasal discharge and nose rubbing were common symptoms of paediatric allergic rhinitis, while nasal congestion and sneezing increased with age. Although there were slight differences depending on the age of patients, one-third to nearly half were believed to have nasal symptoms and asthma attacks. The first wheezing episode preceded nasal symptoms in the majority of patients, but onset of allergic rhinitis occurred > 1 year before asthma in a little over 10%. The age of onset of allergic rhinitis was 1 year in these patients, which was clearly earlier than that observed in patients who first developed asthma (4 years). Furthermore, the incidence of cedar pollinosis was higher in patients who developed allergic rhinitis first than in those who developed asthma first, suggesting that asthma patients who develop allergic rhinitis first exhibit pronounced nasal hypersensitivity. Allergic inflammatory cells are present in the nasal mucosa of asthma patients who do not exhibit distinct allergic rhinitis symptoms, so nasal sensitization does not necessarily lead to allergic rhinitis symptoms, suggesting the existence of some other factor that induces nasal hypersensitivity. On nasal smear, the appearance of mast cells preceded a positive radioallergosorbent test (RAST), and the appearance of eosinophils and basophils was consistent with the degree of sensitization. Anti-allergic agents appear efficacious in patients' mast cells positive on nasal smear cytology with negative house dust mite RAST, suggesting that nasal smear cytology may be useful when beginning medication.

Keywords allergic march, allergic rhinitis, asthma, cedar pollinosis, cytology, questionnaire, nasal swab, RAST, wheezing

Introduction

Many children with bronchial asthma also have allergic rhinitis [1]. When allergic rhinitis is very active, airway hyperresponsiveness increases, often aggravating the symptoms of bronchial asthma. Furthermore, the onset of allergic rhinitis sometimes precedes bronchial asthma, and onset of bronchial asthma may be prevented by successful treatment of allergic rhinitis [2]. Recently, the concept of 'one way, one disease' has drawn attention to the relationship between allergic rhinitis and bronchial asthma in terms of the mechanism of airway inflammation, selection of medication and when to start treatment.

Correspondence: Naoki Shimojo, Department of Pediatrics, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chiba, Chuo-ku 260–8670, Japan.
E-mail: shimojo@faculty.chiba-u.jp

Prevention of asthma onset by successful treatment of allergic rhinitis using drugs or allergen-specific hyposensitization therapy has been reported overseas [3, 4]. The relative timing of allergic rhinitis and asthma onset, association between allergic rhinitis and aggravation of asthma and seasonality of allergic rhinitis in asthma patients are important issues when considering early asthma intervention. However, there are few relevant reports of studies conducted in children in Japan [5]. Hence we conducted a randomized questionnaire survey of bronchial asthma patients at medical institutions associated with our institution to investigate the incidence of paediatric bronchial asthma and allergic rhinitis and the relative timing of onset of either disease. In addition, we performed nasal smear cytology as a marker of inflammation in paediatric bronchial asthma patients who did not exhibit distinct nasal symptoms to assist in determining the most appropriate anti-allergic inflammation therapy, and observed allergic inflammatory cell dynamics in nasal mucosa.

Patients and methods

Questionnaire survey of paediatric bronchial asthma patients

The questionnaire consisted of four questions covering the following: (1) whether patients experience sneezing, nose rubbing, watery nasal discharge, or nasal congestion for ≥2 weeks in the absence of having a cold; (2) when their first wheezing episode and nasal symptoms appeared; (3) seasonality of nasal symptoms; and (4) the relationship between their nasal symptoms and asthma. Subjects were the guardians of paediatric bronchial asthma patients aged 1–18 years.

Nasal smear cytology

Subjects were 39 paediatric bronchial asthma patients aged 1–12 years and not exhibiting distinct allergic rhinitis symptoms. Nasal smear cytology and grading of cell counts were performed using previously reported methods [6].

Results

Questionnaire survey

A total of 248 subjects completed questionnaires. Of these, 205 subjects responded in the affirmative for having ≥1 of sneezing, watery nasal discharge, nasal congestion and nose rubbing. Of these, patients with nasal congestion alone were excluded from the analysis since they may have had a different disorder such as sinusitis, leaving a total of 199 patients (80.2%) believed to have allergic rhinitis. The overall breakdown of nasal symptoms was as follows: 72%, 70%, 62% and 56% experienced nasal congestion, watery nasal discharge, nose rubbing and sneezing, respectively (including multiple responses) (Fig. 1). By age bracket, nose rubbing and watery nasal discharge were most common in younger children, while nasal congestion was most common in those in junior high school and older (data not shown). Looking at the association between nasal symptoms and asthma attacks by the age groups 0–3, 4–6, 7–12 and 13+ years, there was little correlation in the 0–3-year group, but 40–50% of respondents in the other age groups tested reported asthma attacks occurring when nasal symptoms were present (Fig. 2). Regarding the seasonality of nasal symptoms, two-thirds of patients were believed to have perennial allergic rhinitis and one-third cedar pollinosis. By age group, there was no major difference in terms of the perennial nature of nasal symptoms, but older patients were more likely to respond positively for 'perennial and particularly bad from February to April' and 'bad only from February to April', and the presence of cedar pollinosis was suspected in about 50% of patients aged ≥13 years and in about 10% of those aged 0–3 years (Fig. 3).

To analyse the age of onset of wheezing and nasal symptoms, we compared the age of onset of the first wheezing episode and that of nasal symptoms in the 60 patients (of a total of 179 who gave this information) who were free of fever during the first wheezing episode.

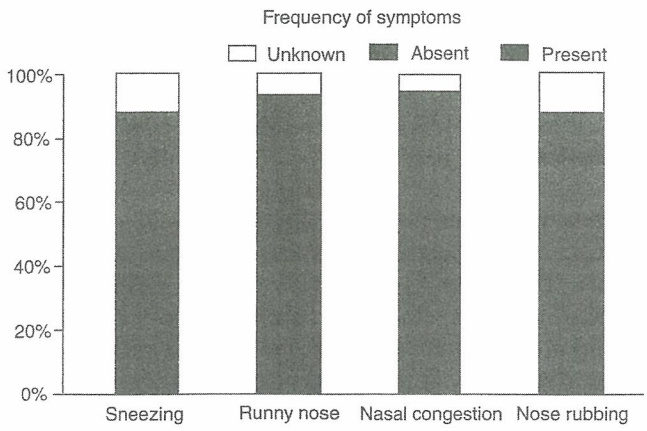

Fig. 1. Nasal symptoms in allergic rhinitis patients aged 1–18 years (n=199).

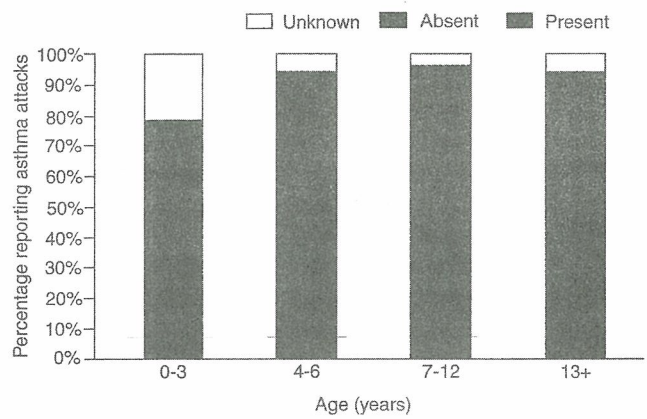

Fig. 2. Presence of experiencing asthma attacks concurrently with having nasal symptoms by age group.

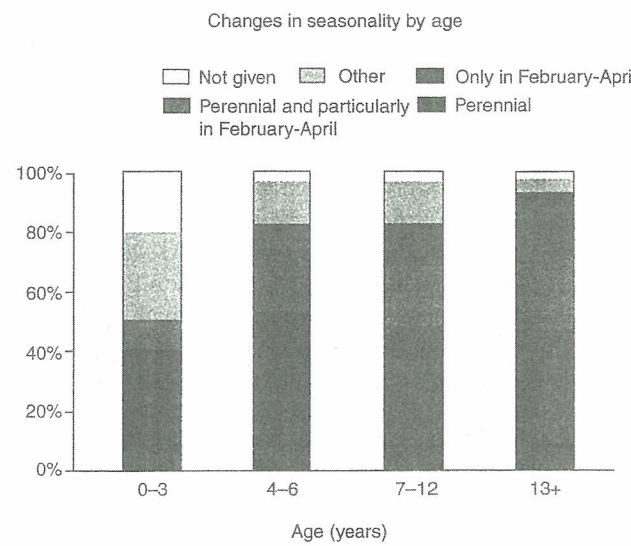

Fig. 3. Seasonality of nasal symptoms by age group.

Twenty-three patients experienced wheezing ≥ 1 year before the appearance of nasal symptoms; 10 experienced nasal symptoms ≥ 1 year before the appearance of wheezing; and the remaining 27 patients experienced both nasal symptoms and wheezing within < 1 year of each other. Overall, wheezing preceded nasal symptoms in most patients (Fig. 4). Similarly, of a total of 119 patients who had fever of $\geq 38.5\,^{\circ}C$ during the first wheezing episode, very few experienced allergic rhinitis before wheezing (results not shown). The average age of onset of asthma was 2 years in patients who developed asthma first and 3.5 years in those who developed allergic rhinitis first or both simultaneously. Nasal symptoms appeared at about age 1 year in patients who developed allergic rhinitis first (Fig. 5). Approximately 70% of patients who developed allergic rhinitis first ($n = 10$) had symptoms of cedar pollinosis, much higher than the number of subjects who developed asthma first (Fig. 6).

Nasal cytology

We performed nasal smear cytology in paediatric bronchial asthma patients who did not exhibit distinct nasal symptoms and analysed mast cell, eosinophil and basophil counts and rates of positive *Dermatophagoides pteronyssinus* radioallergosorbent tests (RAST). Even though nasal symptoms were absent, allergic inflammatory cells were found in the nasal mucosa. Looking at the association with house dust mite sensitization, while mast cells were detected by nasal smear in the majority of asthma patients even if house dust mite RAST was negative, very few eosinophils and basophils were detected. On the other hand, when house dust mite RAST was positive and there was definite sensitization, both eosinophils and basophils appeared on nasal smear. Mast cells also increased with sensitization (Fig. 7). Results obtained in a typical patient are shown in Fig. 8.

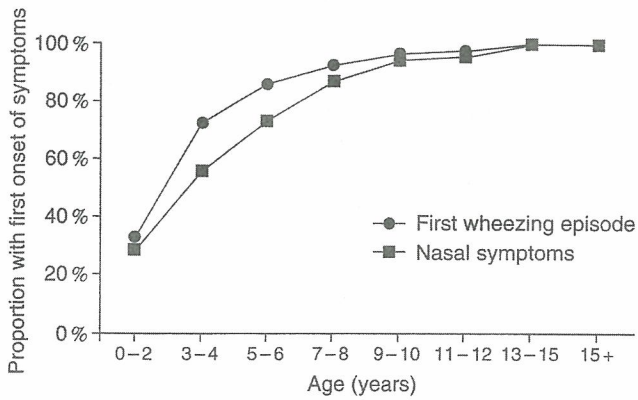

Fig. 4. Age of onset of first wheezing episode and nasal symptoms in patients free of fever during the first wheezing episode ($n = 60$).

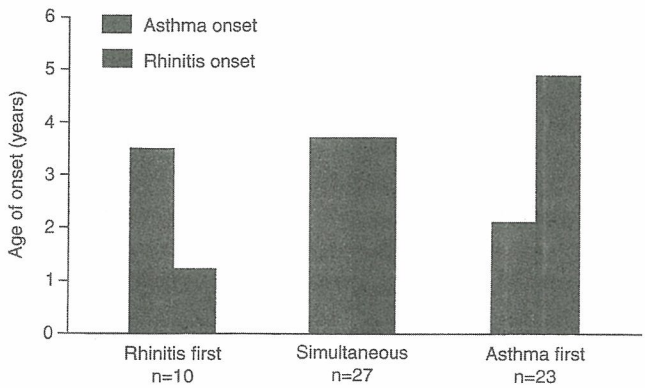

Fig. 5. Relationship between relative timing of allergic rhinitis and asthma onset and age of onset of each disorder in patients with no fever during first wheezing episode ($n = 60$).

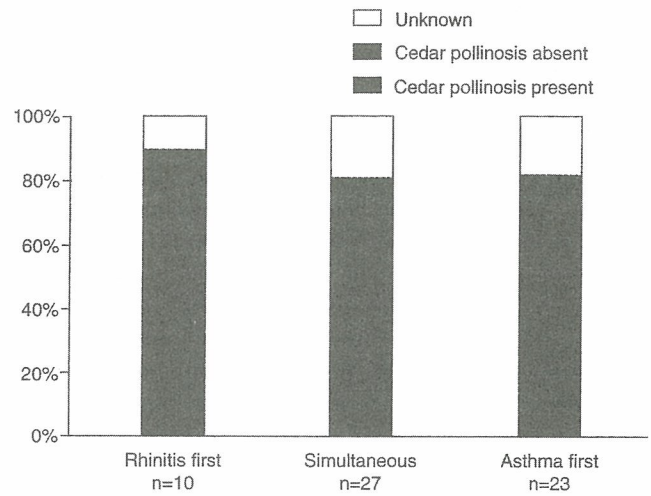

Fig. 6. Relationship between timing of allergic rhinitis and asthma onset and incidence of cedar pollinosis in patients with no fever during first wheezing episode ($n = 60$).

Patients with only mast cells present on nasal smear but exhibiting negative house dust mite RAST who were given ketotifen (0.06 mg/kg/day) for 2 years, eosinophil count disappeared after increasing temporarily. Figure 9a shows the results from one such patient. However, treatment with oxatomide (1 mg/kg/day for 2 years) did not result in any change in eosinophil count in those patients with strongly positive RAST and continuous presence of eosinophils. Results from a typical patient are shown in Fig. 9b.

Discussion

In the present questionnaire survey, 80% of paediatric bronchial asthma patients also had symptoms of allergic rhinitis. This result is very close to the conventionally accepted incidence of allergic rhinitis in paediatric asthma patients in Japan based on diagnoses by doctors [1], suggesting good reliability of the results of this questionnaire. The survey also suggests that, unlike overseas [7], there are few paediatric asthma patients in Japan who develop allergic rhinitis before asthma. The reason for this is not clear, but

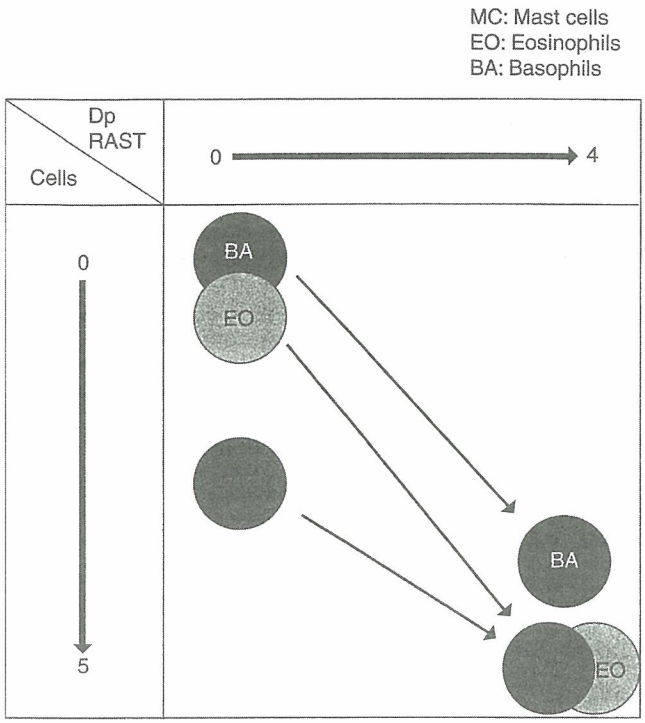

Fig. 7. Changes in house dust mite RAST antibodies and nasal smear cell groups.

the allergens in asthma patients overseas may differ from the main allergen in Japan, house dust mites. In Japan, house dust mites are a major allergen common to both allergic rhinitis and asthma. The allergic rhinitis symptoms in two-thirds of the paediatric asthma patients in the present study were perennial, suggesting that house dust mites are involved to a large extent.

An interesting result of this survey was that while allergic rhinitis symptoms began at around age 1 year in patients who developed allergic rhinitis first, nasal symptoms first appeared after 4 years of age in those who developed asthma first. Given that a higher percentage of patients who developed allergic rhinitis first had cedar pollinosis compared with among those who developed asthma first,

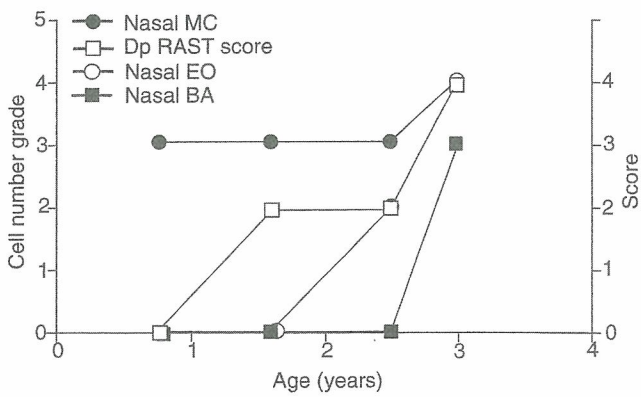

Fig. 8. Changes in house dust mite RAST antibodies and nasal smear cell groups over time in a typical asthma patient.

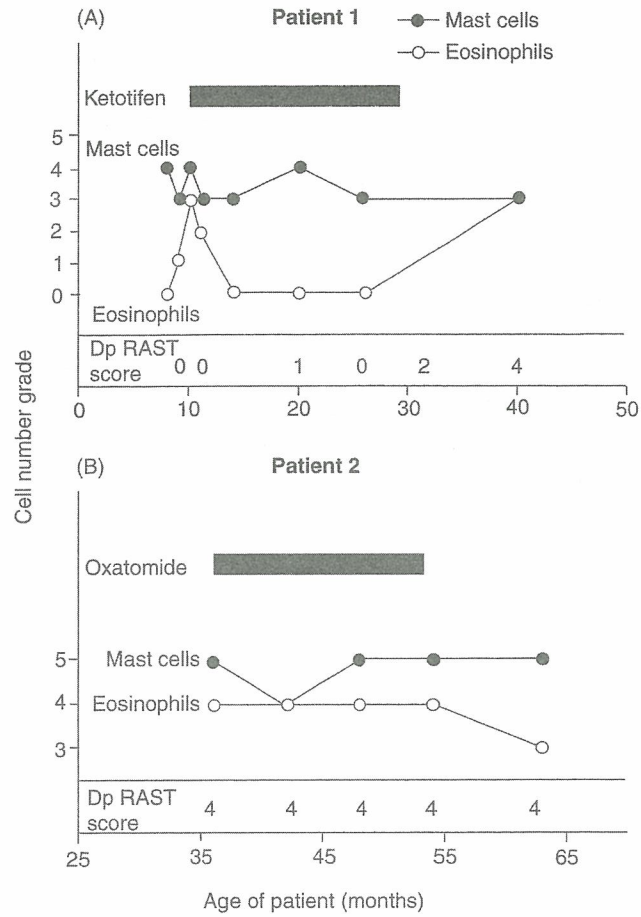

Fig. 9. Changes in house dust mite RAST antibodies and nasal smear cell groups (mast cells and eosinophils) over time in two bronchial asthma patients given (a) ketotifen (0.06 mg/kg/day for 2 years) and (b) oxatomide (1 mg/kg/day for 2 years).

the more pronounced the nasal hypersensitivity, the earlier the onset of allergic rhinitis occurred, indicating a possible susceptibility to cedar pollen sensitization. Additionally, the observation that >90% of paediatric bronchial asthma patients are sensitized to house dust mites and many have perennial allergic rhinitis suggests that the incidence of cedar pollinosis may increase when house dust mite-induced allergic rhinitis is present.

There are two possible explanations for the presence of mast cells, eosinophils and basophils on nasal smear in bronchial asthma patients who do not have distinct nasal symptoms. One is that these individuals have mild allergic rhinitis without clear clinical symptoms, and the other is that they are sensitized but develop only asthma and not allergic rhinitis. In support of the latter explanation, a big difference in nasal hypersensitivity has been observed between paediatric bronchial asthma patients with and without allergic rhinitis (A. Konno, personal communication 2003). In future, hypersensitivity testing of the nasal mucosa of asthma patients both with and without pronounced nasal symptoms needs to be carried out, together with analysis of the types and numbers of allergic

inflammatory cells and cellular activation. Our initial results suggest that nasal smear cytology may be an indicator of local sensitization, and this may in turn help determine when to give the patient anti-allergic agents. However, further investigation with a larger sample of patients is necessary to clarify this assumption.

References

1 Okuda M. Nasal Allergies – Basic and Clinical Research. Tokyo: Iyaku Journal Co. Ltd, 1999 [in Japanese].

2 Bousquet J, Van Cauwenberge P, Khaltaev N. Allergic rhinitis and its impact on asthma. J Allergy Clin Immunol 2001; **108** (Supplement):147–334.

3 Corren J, Adinoff AD, Buchmeier AD, Irvin CG. Nasal beclomethasone prevents the seasonal increase in bronchial responsiveness in patients with allergic rhinitis and asthma. J Allergy Clin Immunol 1992; **90**:250–6.

4 Moller C, Dreborg S, Ferdousi HA et al. Pollen immunotherapy reduces the development of asthma in children with seasonal rhinoconjunctivitis (the PAT-study). J Allergy Clin Immunol 2002; **109**:251–6.

5 Iikura Y, Tokuda K, Koya N et al. Current state of allergy disorder therapy. Allergic march prevention campaign. Shonika-Shinuyo 1991; **54**:1109–13 [in Japanese].

6 Hirano K, Shimojo N, Saito T et al. Nasal cytology in treatment of bronchial asthma – investigation of association between mast cells, eosinophils and basophils on nasal smear cytology and specific IgE antibodies to house dust mites as assayed by RAST. Arerugi 1995; **44**:1117–24 [in Japanese].

7 Pedersen PA, Weeke ER. Asthma and allergic rhinitis in the same patients. Allergy 1983; **38**:25–9.

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 22, Issue of May 28, pp. 23123–23133, 2004
Printed in U.S.A.

CD28 Costimulation Controls Histone Hyperacetylation of the Interleukin 5 Gene Locus in Developing Th2 Cells*

Received for publication, February 4, 2004, and in revised form, March 22, 2004
Published, JBC Papers in Press, March 23, 2004, DOI 10.1074/jbc.M401248200

Masamichi Inami‡, Masakatsu Yamashita‡, Yoshiyuki Tenda‡, Akihiro Hasegawa‡, Motoko Kimura‡, Kahoko Hashimoto§, Nobuo Seki¶, Masaru Taniguchi‖, and Toshinori Nakayama‡**

From the ‡Department of Immunology, Graduate School of Medicine, Chiba University, 1-8-1 Inohana Chuo-ku, Chiba 260-8670, §Department of Life and Environmental Sciences and High Technology Research Center, Chiba Institute of Technology, Narashino, Tsudanuma, Chiba 275-0016, ¶Exploratory Research Laboratories, Department of Bioscience, Fujisawa Pharmaceutical Co. Ltd., 5-2-3, Tokodai, Tsukuba, Ibaraki 300-2698, and ‖Laboratory for Immune Regulation, RIKEN Research Center for Allergy and Immunology, Yokohama 230-0045, Japan

Interleukin 5 (IL-5) plays a unique role in allergic inflammatory responses, and the understanding of molecular mechanisms underlying the generation of IL-5-producing cells is crucial for the regulation of allergic disorders. Differentiation of naive CD4 T cells into type-2 helper (Th2) cells is accompanied by chromatin remodeling including hyperacetylation of histones H3 and H4 in the nucleosomes associated with the IL-4, IL-13, and IL-5 genes. Histone hyperacetylation of the IL-5 gene displayed a delayed kinetics compared with that of the IL-4 and IL-13 genes, suggesting a distinct remodeling mechanism for the IL-5-gene locus. Here we studied the role of CD28 costimulation in the generation of IL-5-producing cells and the histone hyperacetylation of the IL-5 gene locus. CD28-costimulation selectively enhanced histone hyperacetylation of the IL-5 gene locus that appeared to be mediated through NF-κB activation and subsequent up-regulation of GATA3. The CD28 costimulation-sensitive histone hyperacetylation spanned almost the entire intergenic region between the IL-5 and RAD50 accompanied with intergenic transcript. Thus, this is the first demonstration that CD28 costimulation controls a chromatin-remodeling process during Th2 cell differentiation.

Upon antigen recognition by T cell receptor (TCR),[1] naive CD4 T cells differentiate into two distinct helper T (Th) cell subsets, Th1 and Th2 cells (1). Th1 cells produce IFNγ and tumor necrosis factor-β and initiate cell-mediated immunity against intracellular pathogens. Th2 cells produce IL-4, IL-5, and IL-13 and are involved in humoral immunity and allergic responses. The cytokine environment is crucial in controlling the direction of Th cell differentiation (2, 3). For Th1 cell differentiation, IL-12-mediated activation of signal transducer and activator of transcription (STAT) 4 is required, whereas IL-4-mediated STAT6 activation is important for Th2 cell generation (4–6). In addition, TCR stimulation events upon encounter with antigens are also indispensable for both Th1 and Th2 cell differentiation. We reported that efficient TCR-mediated activation of the $p56^{lck}$, calcineurin, and Ras-extracellular signal-regulated kinase mitogen-activated protein kinase signaling cascade is crucial for Th2 cell differentiation (7–9). Recent studies have identified several transcription factors that control Th1/Th2 cell differentiation (10). Among them, GATA3 appears to be a master transcription factor for Th2 cell differentiation. GATA3 is selectively induced in developing Th2 cells, and the ectopic expression of GATA3 induced Th2 cell differentiation even in the absence of IL-4 or STAT6 (11–14). For Th1 cell differentiation, T-bet was recently identified as a key transcription factor (15).

CD28 costimulation enhances Th2 responses significantly (16, 17). Upon anti-CD28 mAb stimulation, phosphatidylinositol 3-kinase is recruited to CD28 and activated, and then subsequent activation of NF-κB is induced (18–21). It has been reported that GATA3 induction was an outcome of the CD28-induced NF-κB activation in T cells (22, 23). This may be a mechanism by which Th2 responses were enhanced by CD28 costimulation. It is also known that IL-5 production and IL-5-dependent airway inflammation are dependent on NF-κB family members (24–26).

Chromatin remodeling of the Th2 cytokine gene loci (IL-4/IL-5/IL-13) occurs during Th2 cell differentiation (27). A highly conserved 400-bp noncoding sequence 1 (CNS1) was identified, and an important role in coordinate expression of Th2 cytokines was revealed (28, 29). More recently, a 3′ distal IL-4 enhancer (V_A) containing an inducible DNase I hypersensitive site was identified (30). Reiner and co-workers (31) report that demethylation of the intron 2 region of the IL-4 gene was associated with cell cycle progression and Th2 cell differentiation (31). We reported that demethylation of this region is regulated by *polycomb* group genes (32) that are known to

* This work was supported by grants from the Ministry of Education, Culture, Sports, Science, and Technology of (Japan) (Grants-in-aid for Scientific Research, Priority Areas Research 13218016 and 12051203 and Scientific Research B 14370107, Advanced and Innovational Research Program in Life Science, and Special Coordination Funds), the Ministry of Health, Labor, and Welfare (Japan) (a grant-in-aid for research on Advanced Medical Technology), the Program for Promotion of Fundamental Studies in Health Science of the Organization for Pharmaceutical Safety and Research (Japan), Human Frontier Science Program Research Grant RG00168/2000-M206, the Hamaguchi Foundation, and the Uehara Memorial Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** To whom correspondence should be addressed: Dept. of Immunology, Graduate School of Medicine, Chiba University, 1-8-1 Inohana Chuo-ku, Chiba 260-8670, Japan. Tel.: 81-43-226-2200; Fax: 81-43-227-1498; E-mail: tnakayama@faculty.chiba-u.jp.

[1] The abbreviations used are: TCR, T cell receptor; Th cells, helper T cells; IFN, interferon; STAT, signal transducers and activators of transcription; IL, interleukin; mAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; CNS1, conserved noncoding sequence 1; ChIP, chromatin immunoprecipitation; GFP, green fluorescent protein; EGFP, enhanced GFP; RT, reverse transcription; IRES, internal ribosome entry site; CREB, cAMP-response element-binding protein.

regulate transcriptional memory in *Drosophila*.

Hyperacetylation of histone H3 and H4 by histone acetyltransferases was suggested to be associated with active chromatin (33). Recently, we and others have demonstrated that histone hyperacetylation of the Th2 cytokine gene loci occurs in developing Th2 cells in a Th2-specific and STAT6-dependent manner (34–36). We demonstrated an essential role for GATA3 in the Th2-specific hyperacetylation (34). We also generated a precise map of the Th2-specific histone hyperacetylation within the IL-13 and IL-4 gene loci and identified a 71-bp conserved GATA3 response element 1.6 kilobase pairs upstream of IL-13 locus exon 1. This histone hyperacetylation remodeling process could be a major target for the Th2 master transcription factor GATA3 to induce differentiation toward Th2 cells.

Histone hyperacetylation of another Th2 cytokine gene locus, IL-5, occurs in a Th2-specific STAT6- and GATA3-dependent manner with significantly different kinetics compared with that of the IL-4 and IL-13 genes (34). The direction of transcription of the IL-5 gene is opposite to that of IL-13 and IL-4. In addition, the RAD50 gene encoding a DNA repair enzyme is located between the IL-5 and IL-13 gene loci. A differential role for GATA3 in the regulation of promoter activity of the IL-5 gene from IL-4 has been suggested (37–39). These results encouraged us to explore possible novel molecular mechanisms that would govern histone hyperacetylation of the IL-5 gene locus.

In the present study we investigated histone hyperacetylation of the IL-5 gene locus in developing Th2 cells cultured with or without CD28 costimulation. A long range CD28-sensitive Th2-specific histone hyperacetylation was detected in the IL-5 and intergenic region of the IL-5 and RAD50 gene loci. The hyperacetylation was accompanied by CD28-sensitive intergenic transcripts and required high expression of GATA3. A molecular mechanism that governs Th2-specific histone hyperacetylation of the IL-5-gene associated nucleosomes will be discussed.

MATERIALS AND METHODS

Mice—C57BL/6 mice were purchased from SLC (Shizuoka, Japan). STAT6-deficient (KO) mice were kindly provided by Shizuo Akira (Osaka University, Osaka, Japan) (40). All mice used in this study were maintained under specific-pathogen-free conditions and were about 4 weeks old. Animal care was in accordance with the guidelines of Chiba University.

Immunofluorescent Staining and Flow Cytometry Analysis—In general, one million cells were stained with appropriate specific antibodies according to a standard method (41, 42). For intracellular staining, fluorescein isothiocyanate-conjugated anti-IFN-γ antibody (XMG1.2; Pharmingen), phosphatidylethanolamine-conjugated anti-IL-4 antibody (11B11; Pharmingen), and allophycocyanin-conjugated anti-IL-5 antibody (TRFK5; Pharmingen) were used. To detect intracellular IL-13, biotinylated polyclonal anti-IL-13 antibody (R&D Systems), and phosphatidylethanolamine-conjugated avidin were used. Flow cytometry analysis was performed on a FACScalibur (BD Biosciences), and results were analyzed by CELLQUEST software (BD Biosciences).

Cell Cultures and in Vitro T Cell Differentiation—Splenic CD4 T cells were stained with anti-CD4-fluorescein isothiocyanate and then purified using magnetic beads and an Auto-MACS sorter® (Miltenyi Biotec), yielding a purity of >98%. Enriched CD4 T cells (1.5×10^6) were stimulated for 2 days with immobilized anti-TCR mAb (H57-597, 3 μg/ml) and soluble anti-CD28 mAb (37.51, 3 μg/ml) in the presence of IL-2 (25 units/ml), IL-12 (100 units/ml), and anti-IL-4 mAb (11B11, 25% culture supernatant) for Th1-skewed conditions. For Th2-skewed conditions, cells were stimulated with immobilized anti-TCR mAb as above but in the presence of IL-2 (25 units/ml), IL-4 (100 units/ml), and anti-IFNγ mAb (R4.6A2, 25% culture supernatant). The cells were then transferred to new dishes and cultured for another 5 days in the presence of immobilized anti-TCR mAb, soluble anti-CD28 mAb, and the cytokines present in the initial culture. To enhance the generation of IL-5-producing cells, stimulation with anti-TCR and anti-CD28 mAbs was performed during the second culture for 5 days. This procedure is slightly different from that used in our previous report (16). Where indicated, wortmannin (Calbiochem) was added to the culture at the doses of 30 or 300 nM for the first 2 days. *In vitro* differentiation was then assessed by intracellular cytokine staining with anti-IL-4, anti-IL-5, anti-IL-13, and anti-IFNγ or by ELISA as described (42).

Chromatin Immunoprecipitation (ChIP) Assay—The ChIP assay was performed using histone H3 ChIP assay kits (17-245: Upstate Biotechnology) as described (34). Anti-GATA3 Ab (H-48: Santa Cruz Biotechnology) was used for precipitation. Where indicated, GFP-positive retrovirus-infected cells were sorted by flow cytometry and subjected to ChIP assay. Several primer sequences for ChIP assay were described previously (34, 42). The primer pairs newly generated are as follows: IL-5 1-F, 5′-$^{-109}$GATTGTTAGCAATTATTCATTTC^{-87}-3′; IL-5, 1-R, 5′-$^{+244}$GGTTAGGACAGCCTACCCTAC^{+224}-3′; IL-5 2-F, 5′-$^{+224}$GTAGGGTAGGCTGTCCTAACC^{+244}-3′; IL-5 2-R, 5′-$^{+646}$GATGCGGGCCATGAGCACATG^{+626}-3′; IL-5 3-F, 5′-$^{+626}$CATGTGCTCATGGCCCGCATC-$^{+646}$-3′; IL-5 3-R, 5′-$^{+1133}$CAGGAGCTTGAGACCTAGAGAG^{+1112}-3′; IL-5 4-F, 5′-$^{+1112}$CTCTCTAGGTCTCAAGCTCCTG^{+1133}-3′; IL-5 4-R, 5′-$^{+1431}$CAACAGAGCTTATATCTCCAGC^{+1452}-3′; IL-5 5-F, 5′-$^{+1452}$GCTGGAGATATAAGCTCTGTTG^{+1431}-3′; IL-5 5-R, 5′-$^{+1795}$GAGAGGCACTAGGATGAAGAG^{+1775}-3′; IL-5 6-F, 5′-$^{+1775}$CTCTTCATCCTAGTGCCTCTC^{+1795}-3′; IL-5 6-R, 5′-$^{+2142}$CTTCAGGAACACCAGACACATAG^{+2120}-3′; IL-5 7-F, 5′-$^{+2142}$CAGATGGTTGTGAGCCAACCATCT^{+2120}-3′; IL-5 7-R, 5′-$^{+2856}$CAAGAAGCAAATAGATCAGACTG^{+2834}-3′; IL-5 8-F, 5′-$^{+2834}$CAGTCTGATCTATTTGCTTCTTG^{+2856}-3′; IL-5 8-R, 5′-$^{+3218}$CTTAAATTGTGAAGTCCTGTCAC^{+3196}-3′; IL-5 9-F, 5′-$^{+3519}$CAAGGCTTTGTGCATGTTACCAAC^{+3542}-3′; IL-5 9-R, 5′-$^{+3919}$CAGTCATGGCACAGTCTGATTC^{+3898}-3′; IL-5-RAD50 1-F, 5′-$^{-15107}$ACTTCACACTGTATGACAGTG^{-15087}-3′; IL-5-RAD50 1-R, 5′-$^{-14691}$CCTGGCTGTGAATGAATATTTGTC^{-14714}-3′; IL-5-RAD50 2-F, 5′-$^{-14336}$ACGCATTGCCCAAATCTTCAG^{-14316}-3′; IL-5-RAD50 2-R, 5′- $^{-13904}$GAAGACGGGTAAGTCCTGAGGCG^{-13925}-3′; IL-5-RAD50 3-F, 5′-$^{-13695}$GAGGACCCAAAAGTTCCGAACACG^{-13672}-3′; IL-5-RAD50 3-R, 5′-$^{-13405}$TTCTAAAAGACAACAGCTACTCGT^{-13428}-3′; IL-5-RAD50 4-F, 5′-$^{-13558}$AGCAACCGTAAGCTCGCACATTCC^{-13585}-3′; IL-5-RAD50 4-R, 5′-$^{-13195}$GACTGCACCCCAGCCTACCCATGC^{-13218}-3′; IL-5-RAD50 5-F, 5′-$^{-13428}$ACGAGTAGCTGTTGTCTTTTAGAA^{-13405}-3′; IL-5-RAD50 5-R, 5′-$^{-13039}$TCACGTGATCAAGAGAAACTACTC^{-13062}-3′; IL-5-RAD50 6-F, 5′-$^{-13218}$GCATGGGTAGGCTGGGGTGCAGTC^{-13195}-3′; IL-5-RAD50 6-R, 5′-$^{-12840}$GACAATCTGCAAGAGCAAGTGCTC^{-12863}-3′; IL-5-RAD50 7-F, 5′-$^{-11433}$CGGAGGATAATTCTCCTTAAAAGG^{-11410}-3′; IL-5-RAD50 7-R, 5′-$^{-11067}$GACAAGGTCTCATTGTAGCTGTGG^{-11090}-3′; IL-5-RAD50 8-F, 5′-$^{-9305}$GCTACATAATGAGTAACCAGCTTG^{-9282}-3′; IL-5-RAD50 8-R, 5′-$^{-8923}$GAGCTGGTGCAGACCTTGTTGCCAC^{-8947}-3′; IL-5-RAD50 9-F, 5′-$^{-7140}$GAGCTGGAAGGTAAAAGGTTAGAATAG $^{-7114}$-3′; IL-5-RAD50 9-R, 5′-$^{-6761}$GAATCTTCAAGGATTGGTGACTATACC $^{-6787}$-3′; L-5-RAD50 10-F, 5′-$^{-3626}$GATTCAGGGAAACAATCTGTGGATG $^{-3612}$-3′; IL-5-RAD50 10-R, 5′- $^{-3300}$GGGTTTATGTGTTCACCATGGACC^{-3323}-3′; IL-5-RAD50 11-F, 5′-$^{-938}$CCTGCACTTTTTGTCATAGGAACC^{-915}-3′; IL-5-RAD50 11-R, 5′-$^{-556}$GTGGTGGTACACACCAGCAGATGTG^{-580}-3′; IL-5-RAD50 14-F, 5′-$^{+3172}$AGGCTGAGGCTGAGCTGCTCCATG^{+3195}-3′; IL-5-RAD50 14-R, 5′-$^{+3549}$TAACTCAGTTGGTAACATGCACAA^{+3526}-3′. The numbers indicate positions relative to the first nucleotide of the IL-5 exon 1, which is designated as +1. The primer pairs for IL-5-RAD50 12 and 13 in Fig. 6 are the same as those for IL-5 promoter and IL-5 intron, respectively.

ELISA—CD4 T cells were cultured for 7 days as described above and restimulated with immobilized anti-TCR mAb (H57-597, 3 μg/ml) for 8 h. The concentrations of IL-5, IL-4, IL-13, and IFNγ in the supernatant were determined by ELISA as described previously (42).

Retroviral Vectors and Infection—pMX-IRES-GFP plasmid was kindly provided by Toshio Kitamura (The University of Tokyo, Tokyo, Japan). The methods for the generation of virus supernatant and CD4 T cell infection were described previously (32). Infected cells were subjected to intracellular staining with anti-IL-4, anti-IL-5, and anti-IFNγ mAb or to cell sorting. A mutant of IκBα, IκBαM, was generated from the IκBα dominant-negative Vector (Mercury). cDNA for human IκBαM or human GATA3 were inserted into a multicloning site of pMX-IRES-GFP.

Immunoblot Analysis—Immunoblot analysis for GATA3, IκBα, and tubulin-α was performed as previously described (42). For IκBα, a rabbit polyclonal Ab (anti-IκBα; Cell Signaling Technology 9242) was used.

RT-PCR—Total RNA was isolated from cultured cells using the TRIzol reagent. Reverse transcription was carried out with Superscript II RT (Invitrogen). 3-Fold serial dilutions of template cDNA were performed. PCR reaction with specific primers was done as described previously (34). New primers used were GATA3 exon 1b forward, CTTTGCGGGATAGTTTAGCAA-3′, and GATA3 exon 1b reverse, 5′-GAA-

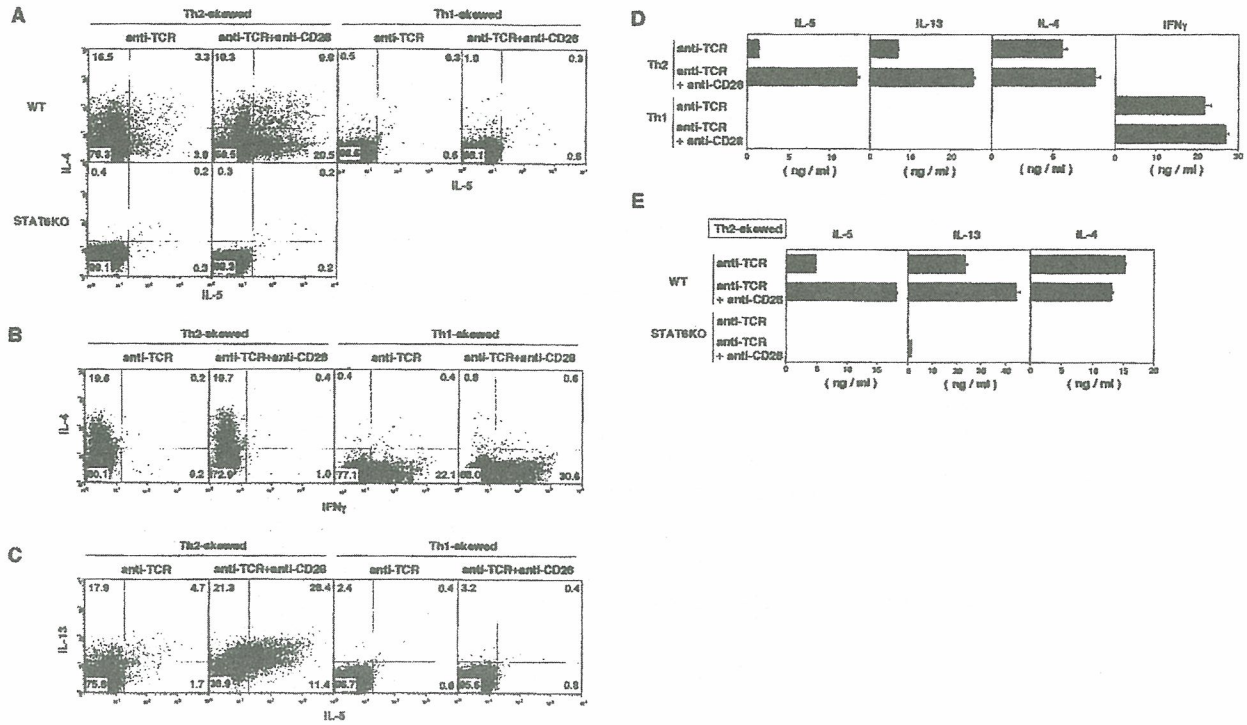

FIG. 1. **Costimulation with anti-CD28 mAb enhanced the generation of IL-5- and IL-13-producing cells.** *A*, *B*, and *C*, freshly prepared splenic CD4 T cells from B6 mice were cultured with immobilized anti-TCR mAb (H57-597, 3 μg/ml) in the presence of costimulation with agonistic anti-CD28 mAb (37.51, 3 μg/ml) under Th1- or Th2-skewed conditions for 7 days. The cultured cells were restimulated, and intracellular IL-5/IL-4, IFNγ/IL-4, and IL-5/IL-13 profiles were determined. The percentages of cells present in the each quadrant are shown. *D* and *E*, CD4 T cells from normal B6 or STAT6-KO mice were cultured under the conditions described above, and restimulation was done with anti-TCR mAb for 8 h. The amounts of IL-5, IL-13, IL-4, and IFNγ in the culture supernatant were measured by ELISA. *WT*, wild type.

CACTGAGCTGCCTGGCGCCGT. A detailed protocol for detection of intergenic transcripts was described previously (34). The primers used are the same as those used in ChIP assay.

RESULTS

Costimulation with Anti-CD28 mAb Enhances the Generation of IL-5- and IL-13-producing Cells—The aim of this study was to clarify the molecular mechanisms that control chromatin remodeling of the IL-5 gene locus during Th2 cell differentiation. We first assessed the role for CD28 costimulation in the generation of IL-5-producing Th2 cells. Freshly prepared CD4 T cells from young adult (4 weeks) B6 mice were cultured *in vitro* with immobilized anti-TCR in the presence of agonistic anti-CD28 mAbs (37.51) to effect stimulation. The IL-5/IL-4 profiles of CD4 T cells cultured under Th1- or Th2-skewed conditions are depicted in Fig. 1*A*. As can be seen, the generation of IL-5-producing cells (both $IL\text{-}5^+IL\text{-}4^-$ and $IL\text{-}5^+IL\text{-}4^+$ fractions) cultured under Th2-skewed conditions was greatly enhanced in the presence of CD28 costimulation. The increased generation of IL-4-producing cells was marginal under these culture conditions. IL-5-producing cells generated with CD28 costimulation were STAT6-dependent and not detected under Th1-skewed culture conditions. The generation of IFNγ-producing Th1 cells was moderately increased by the presence of CD28 costimulation (Fig. 1*B*). The levels of IL-13-producing cells were also increased by the presence of CD28 costimulation (Fig. 1*C*).

Concurrently, the amount of cytokines produced by developing Th2 cells cultured with CD28 costimulation was assessed by ELISA (Fig. 1, *D* and *E*). As expected, CD28 costimulation significantly enhanced the production of IL-5 and IL-13, whereas the effects on the production of IL-4 and IFNγ were marginal. The production of Th2 cytokines (IL-5, IL-13, and IL-4) was all STAT6-dependent regardless of the presence or absence of CD28 costimulation (Fig. 1*E*). Taken together these results suggest that the generation of IL-5- and IL-13-producing cells was Th2-specific, STAT6-dependent, and more sensitive to CD28 costimulation as compared with that of IL-4-producing cells.

Dynamics of Histone H3 Hyperacetylation of the IL-5 Gene Locus in Developing Th2 Cells Cultured with CD28 Costimulation—To clarify whether CD28 costimulation enhances histone hyperacetylation of the IL-5 gene locus during Th2 cell differentiation, we first examined the kinetics of acetylation of the IL-5 promoter, IL-4 promoter, and RAD50 promoter regions using a ChIP assay with anti-acetylhistone H3 Ab. Developing Th1 and Th2 cells cultured with CD28 costimulation were harvested 2, 3.5, 5, and 7 days after stimulation. The results of this analysis are presented as the relative band intensity of each group normalized with band intensity of the corresponding input DNA as shown in the *right panel* of Fig. 2. Two days after stimulation, low but significant levels of increase in acetylation occurred at all of the regions tested, and basically no difference between the three culture conditions was noted. However, as shown in Fig. 2*A*, the levels of histone acetylation associated with the IL-5 promoter increased significantly after cultivation for more than 3 days under Th2 skewed conditions, particularly in those cultures with CD28 costimulation. In contrast, the levels of histone hyperacetylation in the IL-4 promoter were increased day by day, and no significant difference was detected in the presence or absence of CD28 costimulation. The levels of histone hyperacetylation in the RAD50 gene promoter region were increased equivalently under these three culture conditions. These results suggest that histone hyperacetylation of the IL-5 gene locus is more sensitive to CD28

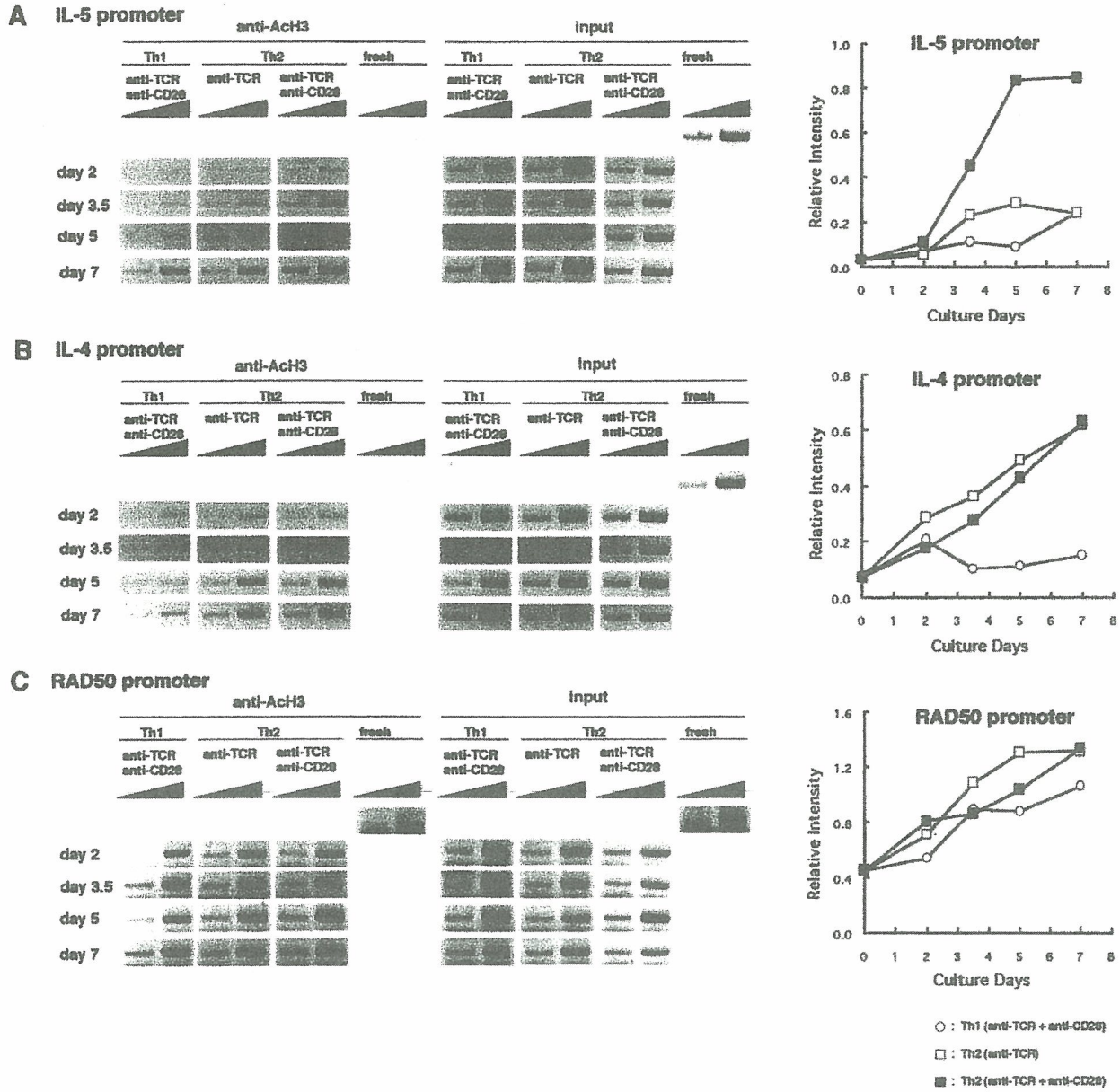

FIG. 2. **Induction of histone H3 hyperacetylation of IL-5, IL-4, and RAD50 gene loci in developing Th2 cells with CD28 costimulation.** Developing Th1 and Th2 cells cultured with immobilized anti-TCR mAb and CD28 costimulation were prepared 2, 3.5, 5, and 7 days after the stimulation. The acetylation status of histone H3 in the nucleosomes associated with the indicated regions was assessed by a ChIP assay with an anti-acetylated histone H3 antibody and specific primer pairs. Before immunoprecipitation for ChIP assay, aliquots of lysates ($\sim 6 \times 10^2$ cell equivalents) were separated for PCR to determine the relative levels of input DNA. 3-Fold serial dilutions were made with both the input DNA and immunoprecipitated DNA samples before PCR. Two independent experiments were performed, and similar results were obtained. Quantitative representations of the results are shown in the *right panels*. The intensities of the bands at the highest concentration were measured by densitometry, and relative intensities (anti-acetyl histone precipitates/input DNA ratio) for each primer pair were calculated.

costimulation than that of the IL-4 locus.

Enhanced Histone Hyperacetylation Induced with CD28 Costimulation Is Observed Only in the IL-5-associated Nucleosomes—Next, we examined the acetylation status of the DNA regions corresponding to IL-5 promoter, IL-5 intron, IL-13 intron, IL-4 promoter, CNS1, IL-4 V_A enhancer, RAD50 promoter, and IFNγ promoter. CNS1 and IL-4 V_A enhancer regions were previously described to contain regulatory elements or DNase I hypersensitive sites (28, 30). Histone hyperacetylation induced in these regions, except for two controls (RAD50 and IFNγ), occurred in a Th2-specific manner as reported previously (34). As can be seen in Fig. 3*A*, the levels of acetylation in the region of IL-5 promoter and IL-5 intron were significantly enhanced by the presence of CD28 costimulation. In contrast, those of other regions were all unaffected. The effect of CD28 costimulation on the histone hyperacetylation within the IL-5 gene loci was analyzed more precisely with a series of primer pairs within the IL-5 genes as shown in Fig. 3*B*. CD28 costimulation significantly enhanced the levels of histone hyperacetylation at all regions within the IL-5 genes tested (Fig. 3*C*).

Next, we assessed the STAT6 dependence of the CD28 costimulation-induced enhancement of the acetylation (Fig. 3*D*). Similar to other regions (IL-13 intron, IL-4 promoter,

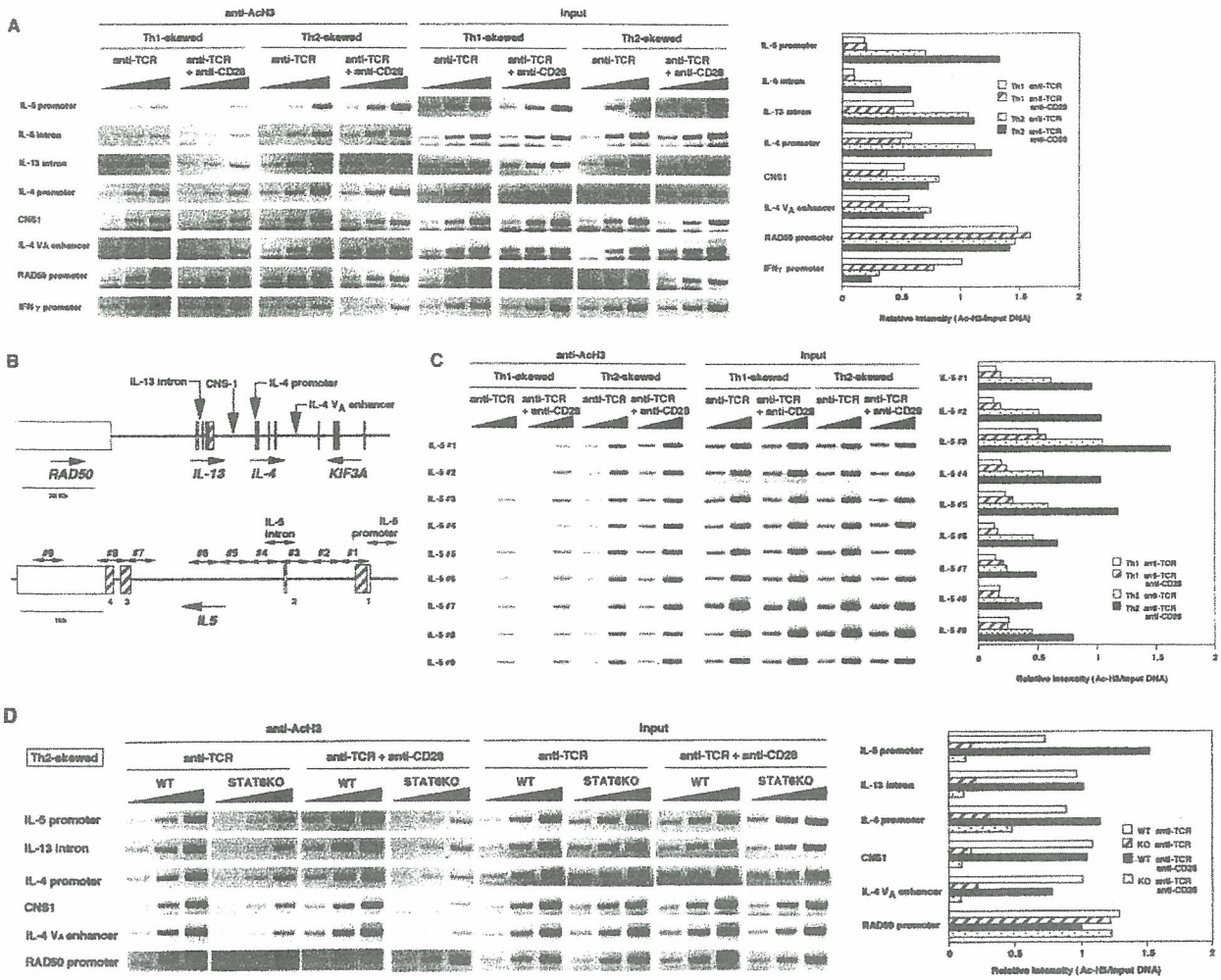

FIG. 3. **Hyperacetylation of histone H3 of the IL-5, IL-13, and IL-4 gene loci in developing Th2 cells cultured with CD28 costimulation.** *A*, developing Th1 and Th2 cells cultured with CD28 costimulation were prepared and subjected to ChIP assays with the indicated primer pairs. 3-Fold serial dilution series were made. The intensity of bands of the highest concentration was measured, and relative intensities are shown in the *right panel*. Similar results were obtained by measuring the bands of intermediate concentration. Three independent experiments with different T cell preparations were performed with similar results. *B*, schematic representation of the IL-5 gene locus and the IL-13 and IL-4 gene loci is shown with the location of the specific primers used in *panel A* and *C*. The location of the primer pairs used in *panel A* is as follows (5′ to 3′). IL-5 promoter F, −521 to −498; IL-5 promoter R, −76 to −101; IL-5 intron F, +857 to +883; IL-5 intron-R, +1214 to +1188. The numbers indicate positions relative to the first nucleotide of the IL-5 exon 1, which is designated as +1. IL-13 intron F, +142/+165; IL-13 intron R, +578 to +555; CNS1 F, +7554 to +7579; CNS1 R, +7767 to +7742. The numbers indicate positions relative to the first nucleotide of the IL-13 exon 1, which is designated as +1. IL-4 promoter F, −661 to −637; IL-4 promoter R, −164 to −187; IL-4 V_A enhancer F, +13234 to +13257; IL-4 V_A enhancer R, +13439 to +13416. The numbers indicate positions relative to the first nucleotide of the IL-4 exon 1, which is designated as +1. RAD50 promoter F, −242 to −217; RAD50 promoter R, −43 to −67. The numbers indicate positions relative to the first nucleotide of the RAD50 exon 1, which is designated as +1. IFNγ promoter F, −3372 to −3349; IFNγ promoter R, −3025 to −3048. The numbers indicate positions relative to the first nucleotide of the IFNγ exon 1, which is designated as +1. *kb*, kilobase. *C*, a ChIP assay with the indicated primer pairs within the IL-5 gene locus was done as in *panel A*. Three independent experiments were performed with similar results. *D*, a ChIP assay with STAT6-KO-developing Th2 cells was done as in *panel A*. Two independent experiments were performed with similar results.

CNS1, and IL-4 V_A enhancer), histone hyperacetylation of the IL-5 promoter locus was not induced in STAT6-KO T cell cultures even in the presence of CD28 costimulation. Thus, STAT6 is critical for the Th2-specific histone hyperacetylation of the IL-5 gene locus induced by anti-TCR stimulation and CD28 costimulation.

Enhanced Production of IL-5 and Histone Hyperacetylation of the IL-5 Gene Locus Induced by CD28 Costimulation Are Dependent on NF-κB Activation—CD28 costimulation is known to induce phosphatidylinositol 3-kinase activation and the subsequent activation of NF-κB. We tested the effect of wortmannin, a phosphatidylinositol 3-kinase inhibitor, on the CD28-induced enhancement of the generation of IL-5-producing cells (43). The generation of IL-5-producing cells in the culture with CD28 costimulation was decreased in the presence of wortmannin in a dose-dependent manner (Fig. 4*A*). In contrast, the generation of IL-4-producing cells was not affected at any doses of wortmannin tested. Concurrently, the effect of wortmannin on the production of cytokines was assessed by ELISA (Fig. 4*B*), and as expected, the enhanced production of IL-5 with CD28 costimulation was sensitive to wortmannin. IL-13 production was also slightly decreased; however, the levels of IL-4 were not changed by the presence of 30 to 300 nM wortmannin.

Next, we assessed the role for NF-κB activation in the CD28-induced enhancement of the generation of IL-5-producing cells and histone hyperacetylation of the IL-5 gene locus. A mutant form of IκBα (IκBαM), which inhibits the NF-κB activation (44), was inserted in an IRES-GFP retroviral construct, and the

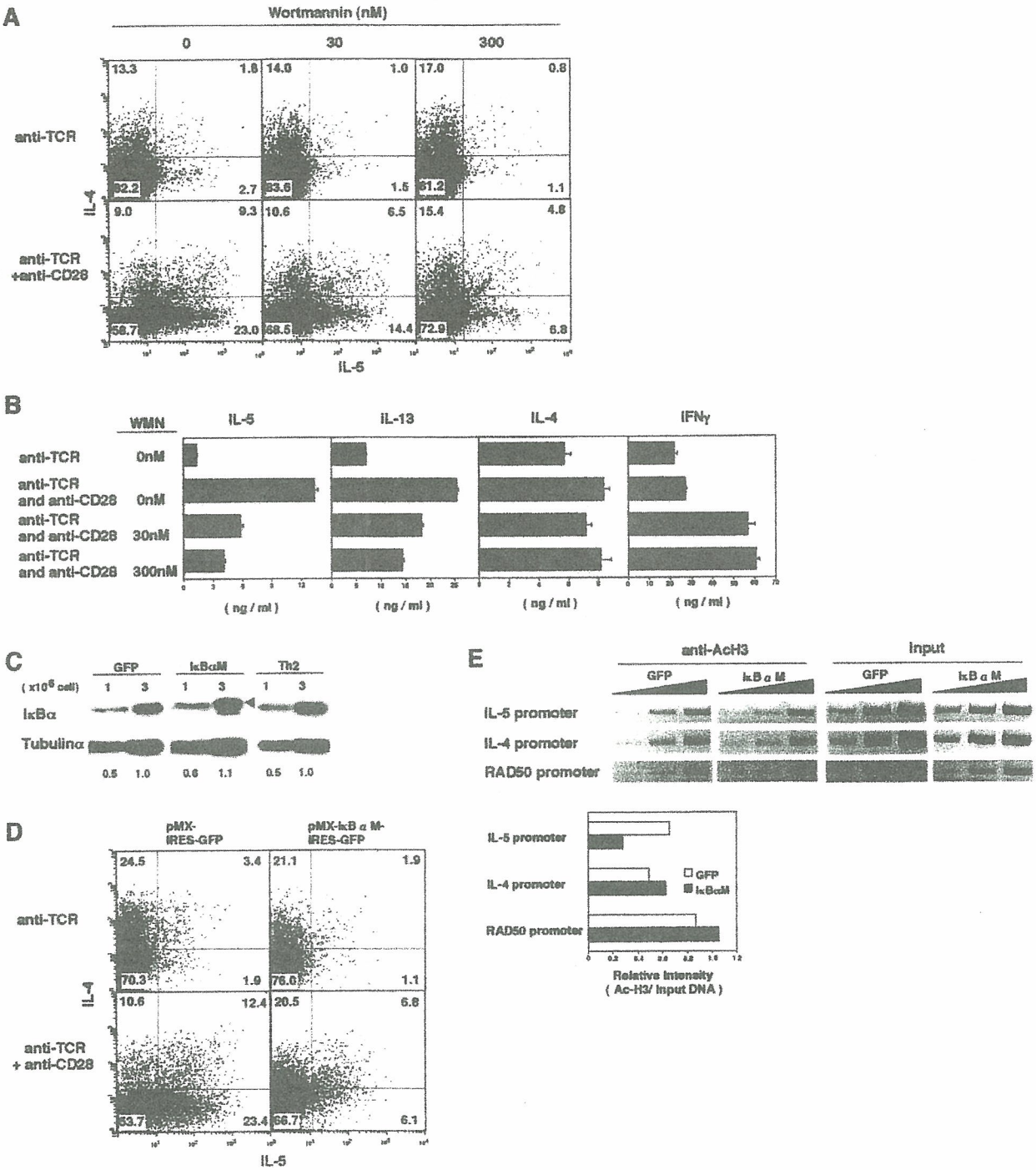

FIG. 4. **Enhanced production of IL-5 and histone hyperacetylation of the IL-5 gene locus induced by CD28 costimulation were dependent on NF-κB activation.** *A*, effect of wortmannin on the generation of IL-5-producing cells under Th2-skewed culture conditions with CD28 costimulation. CD4 T cells were cultured under the conditions described in Fig. 1 in the presence of the indicated doses of wortmannin. The intracellular IL-5/IL-4 profiles are shown. The *numbers* represent the percentages of the cells present in each quadrant. *B*, cytokine production profiles of the cells prepared under the same condition as described in *panel A* are shown. *C*, freshly prepared splenic CD4 T cells were stimulated with anti-TCR and anti-CD28 and infected with a retrovirus pMX-IRES-GFP (*GFP*) or pMX-IκBα-M-IRES-GFP encoding a mutant form of IκBα (*IκBαM*). Seven days after infection GFP^+ cells were sorted and subjected to immunoblotting with an anti-IκBα Ab that reacts with both wild type IκBα and IκBαM. The position of IκBαM is indicated by an *arrowhead*. Also non-infected Th2 cells (Th2) were included. Tubulin α was used as a loading control. *D*, freshly prepared splenic CD4 T cells were stimulated under the indicated conditions and infected with a retrovirus encoding a mutant form of IκBα (*IκBαM*) with EGFP. Seven days after infection, the cultured cells were restimulated, and intracellular IL-5/IL-4 profiles of electronically gated GFP positive populations were determined. The percentages of cells present in the each quadrant are shown. *E*, effect of ectopic expression of IκBαM on histone H3 hyperacetylation in IL-5, IL-4, and RAD5 gene loci. Retrovirus-infected CD4 T cells were prepared as described in *panel C*. One million GFP^+ infected cells were then collected by cell sorting and the acetylation status of histone H3 was determined by ChIP assay. Relative band intensities measured by densitometry are shown in the *right*. At least three independent experiments were done in each (*A–D*) with similar results.