Published December 18, 2006

the Th1/Th2 ratio because bacterial components stimulate Th1 responses that in turn inhibit Th2 responses and IgE production (12). On the other hand, recent findings have indicated that a spectrum of T cells with immunoregulatory properties is involved in the regulation of IgE production and the pathophysiology of allergic diseases (13). For example, $CD4^+CD25^+$ regulatory T cells inhibit Th2 responses by producing immunosuppressive cytokines that can directly inhibit B cell activation (14, 15). Furthermore, NKT cells expressing an invariant antigen receptor (Vα14-Jα281 for mice and Vα24-JαQ for humans; reference 16) suppress Th2 and IgE responses via their production of IFN-γ (17).

In addition to these cellular mechanisms, it has also been reported that IL-21 is involved in the suppression of IgE production in both mice and humans (18, 19). IL-21 is a type I cytokine produced by activated $CD4^+$ T cells and has a broad capacity to regulate lymphoid cell functions (20–22). Among these functions, IL-21 directly inhibits antibody production by IgE-bearing B (Bε) cells induced by CD40L and IL-4 (18). Conversely, IL-21R–deficient mice exhibit enhanced IgE production (23). IL-21 has been shown to specifically inhibit germ line transcription of the IgE constant region (Cε) gene but not of other isotype genes (18). However, there is no direct evidence that this inhibition of germ line transcription is responsible for the suppression of IgE production, as class switch recombination of Ig genes and subsequent antibody secretion are differentially regulated events (24). IL-21 also induces apoptosis in B cells (25, 26), which could partially explain the reduction of IgE production; however, this effect was not shown to be specific for IgE. Hence, the mechanism by which IL-21 specifically inhibits IgE production is not yet fully understood.

Here, we have investigated BCG-mediated IgE suppression and found that NKT cells specifically induced apoptosis in Bε cells through the production of IL-21, resulting in a dramatic decrease in IgE production. IL-21 increased the formation of a complex between Bcl-2 and the proapoptotic molecule Bcl-2–modifying factor (Bmf), which is selectively expressed in Bε cells and counteracts the antiapoptotic activity of Bcl-2. We have found that similar mechanisms are operative in humans. This is the first report demonstrating that IL-21 produced by Vα14 NKT cells plays an important role in the regulation of IgE responses in both mouse and human immune systems.

RESULTS

Vα14 NKT cell–dependent IgE suppression by BCG treatment

We used an OVA-patched sensitization protocol (27) to determine if BCG activates Vα14 NKT cells. Vα14 NKT cells were detected by α-galactosylceramide (α-GalCer)–loaded CD1d tetramer staining. In control mice treated with PBS or OVA without BCG, ~15% of the liver mononuclear cells (MNCs) were Vα14 NKT cells (Fig. 1 A, left and middle). However, BCG treatment significantly increased the frequency of Vα14 NKT cells to >25% (Fig. 1 A, right). BCG treatment also increased the absolute number of Vα14 NKT cells because the total number of liver MNCs was also increased by 50–80% (not depicted). Sera were collected from these mice 1 wk after the last sensitization, and IgE levels were evaluated. In WT mice, both total and OVA-specific IgE levels were suppressed by BCG treatment (Fig. 1 B). In mice lacking the Jα18 gene (Vα14 NKT KO), there was no significant BCG-induced suppression of IgE responses, suggesting that suppression requires Vα14 NKT cells.

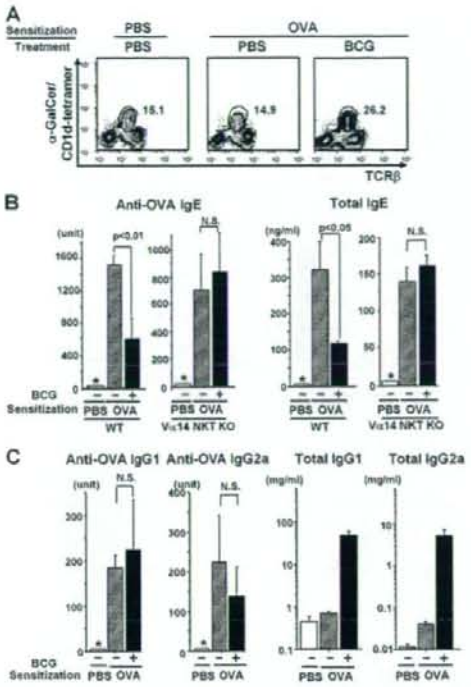

Figure 1. Requirement of Vα14 NKT cells in BCG-mediated IgE suppression. (A) FACS profiles of liver MNCs. The liver MNCs obtained 1 wk after the last immunization were stained with α-GalCer/CD1d tetramer and anti-TCRβ mAb. Three mice per each group were analyzed and representative data are shown. (B and C) Effects of BCG on antibody responses in WT and Vα14 NKT KO mice. Total and OVA-specific serum IgE (B), IgG1, and IgG2a (C) were assayed by ELISA. Five mice were used in each group. Values are expressed as mean ± SD. The asterisks (*) indicate that the amount of IgE was below the detection level for anti-OVA IgE (<31.2 U/ml), anti-OVA IgG1 (<0.002 U/ml), or anti-OVA IgG2a (<1.25 U/ml). N.S., not significant. All experiments were repeated three times with similar results.

The effect of BCG administration on Th1/Th2 responses

It is well known that the isotype commitment of B cells during Ig class switching is tightly regulated by Th1/Th2 cell cytokines (28) and that Vα14 NKT cells play a regulatory role in T cell differentiation (17, 29, 30). Therefore, we measured serum IgG2a (Th1) and IgG1 (Th2) levels to assess any changes in the Th1/Th2 balance. BCG administration did

Published December 18, 2006

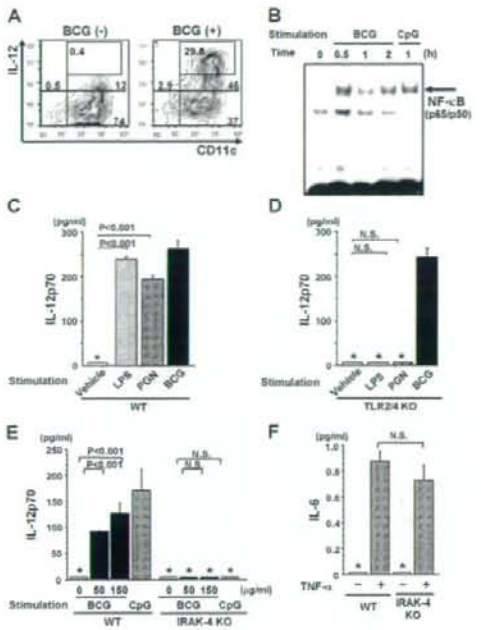

Figure 2. Activation of DCs by BCG. IL-12 production (A) and NF-κB activation (B). (A) Intracellular staining of BM-DCs with anti-IL-12p40/p70 and anti-CD11c mAbs with or without in vitro BCG (50 μg/ml) treatment for 12 h. BCG-treated BM-DCs (10,000 cells) were analyzed by FACS, and the number in each panel indicates the percentage of total cells. (B) NF-κB activation. 2×10^5 BM-DCs were stimulated with or without 50 μg/ml BCG or 1 μM CpG in vitro. NF-κB activity was determined by EMSA. (C and D) No requirement of TLR2 and TLR4 in BCG-mediated IL-12 production. 2×10^5 BM-DCs derived from WT (C) or TLR2/4 double KO (D) mice were stimulated in vitro with or without 10 μg/ml LPS, 10 μg/ml PGN, or 150 μg/ml BCG for 48 h, and IL-12p70 levels were measured by ELISA. (E and F) Requirement of IRAK-4 for IL-12 production. 2×10^5 BM-DCs were assayed for IL-12p70 by ELISA after stimulation with 0, 50, or 150 μg/ml BCG or 1 μM CpG (E), and for IL-6 with 10 ng/ml TNF-α stimulation for 48 h (F). In C–F, values are expressed as mean ± SD of triplicate cultures. The asterisks (*) indicate that the levels were below the detection limits for IL-12p70 (<62.5 pg/ml) and IL-6 (<15.6 pg/ml). N.S., not significant. All experiments were repeated twice with similar results.

not significantly alter the levels of OVA-specific IgG1 or IgG2a, although total levels of both isotypes were significantly enhanced (Fig. 1 C).

Innate signaling pathway for BCG-mediated IL-12 production

During microbial infection, both CD1d- and IL-12–mediated signals are required for the rapid activation of Vα14 NKT cells (31). Thus, we assessed IL-12 production after BCG treatment. BM-derived DCs (BM-DCs) were stimulated in vitro with 50 μg/ml BCG and examined for IL-12 production by intracellular cytokine staining using an IL-12p40/p70 mAb. Upon BCG stimulation, a large fraction of $CD11c^{high}$ cells

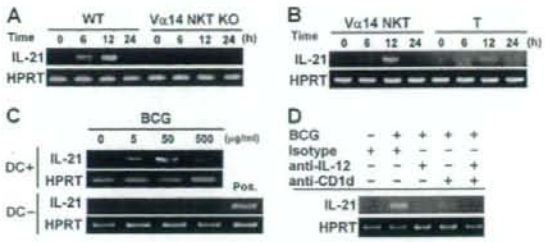

Figure 3. IL-21 expression. (A) Vα14 NKT cell–dependent IL-21 production. Liver MNCs were obtained after BCG injection (500 μg/mouse) and examined for IL-21 mRNA expression. (B) Identification of the source of IL-21. Vα14 NKT and conventional T cells were sorted from liver MNCs and examined for IL-21 mRNA expression. (C) Requirement of DCs for BCG-induced IL-21 expression by Vα14 NKT cells. Liver $TCR\beta^+$ cells were cultivated in the presence of 50 μg/ml BCG with (top) or without (bottom) BM-DCs for 24 h and analyzed for IL-21 mRNA expression. Liver $TCR\beta^+$ cells stimulated with 10 μg/ml anti-CD3 mAb were used as a positive (Pos.) control. (D) Requirement of IL-12– and CD1d-mediated signals for IL-21 mRNA expression upon BCG stimulation. An isotype control, anti-IL-12p40/p70, or anti-CD1d mAb (20 μg/ml) was added to the cultures of liver $TCR\beta^+$ cells and BM-DCs as described in C. All experiments were repeated twice with similar results.

produced IL-12 (Fig. 2 A). NF-κB activation is crucial for IL-12 production, and BCG treatment activated NF-κB to the same extent as treatment with the positive control CpG, as demonstrated by electrophoretic mobility shift assay (Fig. 2 B). These results indicate that BCG directly induces IL-12 production in DCs by activating NF-κB.

It has been reported that mycobacterial cell wall antigens such as peptidoglycan (PGN) or lipoarabinomannan induce proinflammatory gene transcription through TLR2 and TLR4 (32). However, when we compared IL-12p70 production by BCG-stimulated WT and TLR2/TLR4 double KO BM-DCs, there was no difference (Fig. 2 C). As expected, however, the TLR2/4-deficient cells failed to respond to LPS or PGN (Fig. 2 D). These results indicate that receptor(s) other than TLR2 and TLR4 are responsible for the recognition of whole BCG organisms.

To analyze intracellular signaling pathways activated by BCG, we measured IL-12p70 production by BM-DCs from WT and IL-1R–associated kinase (IRAK)-4 KO mice. BM-DCs from IRAK-4 KO mice produced less IL-12p70 than those from WT mice in response to both BCG and CpG (Fig. 2 E), whereas they produced comparable levels of IL-6 in response to TNF-α stimulation (Fig. 2 F). Similarly, BM-DCs from myeloid differentiation factor 88 (MyD88) KO mice produced nearly undetectable IL-12p70 upon BCG stimulation, whereas IL-6 production remained unchanged (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20062206/DC1). Therefore, the recognition of BCG organisms is mediated by innate receptors other than TLR2 and TLR4 that signal through both IRAK-4 and MyD88.

Published December 18, 2006

BCG-induced IL-21 expression in Vα14 NKT cells

The recently identified IL-21 and its receptor (IL-21R), members of the common γ-chain (γc)-dependent cytokine family, have been shown to regulate IgE production without influencing Th2 cell differentiation (18, 20, 23). Thus, we examined the possibility that IL-21 might be induced by BCG stimulation and might suppress IgE responses in a Vα14 NKT cell–dependent manner. We first measured IL-21 mRNA expression in TCRβ$^+$ liver MNCs by a RT-PCR. IL-21 mRNA was detected in liver TCRβ$^+$ liver MNCs of WT mice within 6 h after BCG injection (Fig. 3 A). In contrast, no IL-21 mRNA was detected in the Vα14 NKT KO mice (Fig. 3 A), suggesting that Vα14 NKT cells are the source of IL-21 in response to BCG. To test this hypothesis, we separated conventional T cells and Vα14 NKT cells and found that IL-21 mRNA was more abundant in the Vα14 NKT cells after BCG injection (Fig. 3 B). Similarly, after stimulation with anti-CD3, IL-21 mRNA levels in Vα14 NKT cells were more than seven times higher than in CD4 T cells, confirming that these cells are the major source of IL-21 in this model (Fig. S2 A, available at http://www.jem.org/cgi/content/full/jem.20062206/DC1).

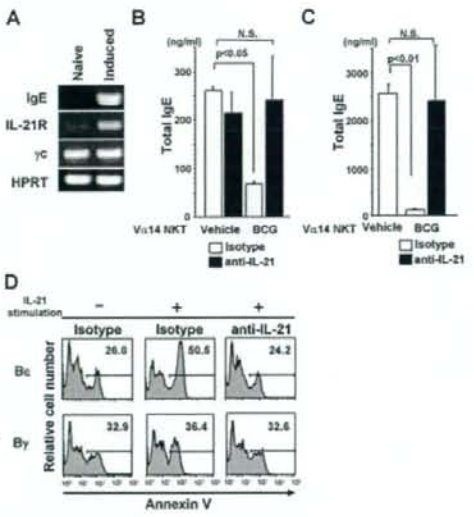

Figure 4. IL-21–mediated Bε cell apoptosis. (A) RT-PCR analysis. Expression of IgE (Cε), IL-21R, and γc was investigated in naive B (left) and Bε (right) cells. (B) Suppression of IgE production in naive B cell cultures. Naive B cells and Vα14 NKT cells (10^5 each) were cocultured in the presence of sCD40L and IL-4. (C) Suppression of IgE production in the Bε cell culture. 10^5 Vα14 NKT cells were added to the Bε cell (10^5) cultures. In B and C, 20 μg/ml anti-IL-21 mAb or isotype control mAb was added at the same time as the Vα14 NKT cells. The concentration of total IgE was measured by ELISA in triplicate. Values are expressed as mean ± SD. N.S., not significant. The experiments were repeated three times with similar results. (D) IL-21–mediated Bε cell apoptosis. 2×10^5 Bε and Bγ cells were generated and then further cultured with or without 30 ng/ml IL-21 for 30 h. Annexin V staining was then performed. The numbers represent percentage of the gated cells. Annexin V$^+$ cells among Bε and Bγ cells just before IL-21 treatment was 25.7 and 29.2%, respectively (not depicted). The experiments were repeated three times with similar results.

Requirement for IL-12 and CD1d in IL-21 expression by Vα14 NKT cells

We next analyzed the role of DCs in BCG-induced IL-21 mRNA expression. Co-culture of Vα14 NKT cells with DCs plus IL-12 strongly induced IL-21 mRNA expression, whereas no IL-21 mRNA was induced in the absence of DCs (Fig. 3 C). Furthermore, IL-21 mRNA expression was inhibited by the addition of anti–IL-12, anti-CD1d, or both into the cultures (Fig. 3 D), indicating that both IL-12 and CD1d are required for IL-21 expression by Vα14 NKT cells.

IL-21–mediated IgE suppression

To examine whether BCG-activated Vα14 NKT cells actually suppress IgE production, Bε cells were generated from naive CD19$^+$ splenic B cells using the 3-d culture system described by Snapper et al. (33). The starting population of naive B cells expressed negligible IL-21R and contained no Bε cells as defined by Cε transcripts (Fig. 4 A). However, after 3 d of the culture, the majority of CD19$^+$ B cells became Bε cells and expressed IL-21R (Fig. 4 A). We then investigated the effects of BCG treatment on B cells, before and after IgE class switching. The addition of BCG-treated liver MNCs at the onset of the naive B cell cultures significantly suppressed IgE production (~50%; Fig. 4 B). However, when BCG-activated Vα14 NKT cells were added to the Bε cell culture on day 3 and the cells were further cultivated for 5 d, IgE production was even more strongly inhibited (>90% suppression; Fig. 4 C). These results indicate that, even after B cells have undergone Cε class switching, BCG-activated Vα14 NKT cells can potently suppress IgE production. The inhibition of IgE production was IL-21 dependent, as an anti–IL-21 mAb completely abrogated the inhibitory effects (Fig. 4, B and C). When the B cells in these cultures were assessed for apoptosis by annexin V staining, there was a significant increase

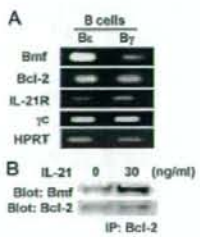

Figure 5. Bmf-mediated Bε cell apoptosis. (A) RT-PCR. RNA from Bε and Bγ cells was analyzed for its expression of the indicated genes by RT-PCR. Note that no significant differences in Bcl-2 and IL-21R expression between Bε and Bγ cells were observed. (B) Western blotting. Bε cells were stimulated with IL-21 at 37°C for 30 min, and their cell lysates (6×10^6) were subjected to immunoprecipitation with anti–Bcl-2 mAb and immunoblotting with anti-Bmf antibody (top) or anti–Bcl-2 mAb (bottom). All experiments were repeated three times with similar results.

Published December 18, 2006

in apoptotic Bε cells that was not observed in the Bγ cells (Fig. 4 D, middle). Apoptosis of Bε cells was abrogated by the addition of anti–IL-21, a treatment that had no significant effect on Bγ cells (Fig. 4 D, right).

Bmf-induced Bε cell apoptosis

To understand the molecular mechanisms underlying IL-21–induced IgE suppression, we performed DNA microarray analyses to compare gene expression between Bε and Bγ cells. The DNA microarray data were deposited in the Center for Information Biology Gene Expression database (CIBEX; http://cibex.nig.ac.jp/) under accession number CBX15. The proapoptotic Bmf gene (34) was dramatically up-regulated in Bε cells, a finding that was confirmed by RT-PCR (Fig. 5 A). No significant difference in the expression of IL-21R, Bcl-2, or γc was detected (Fig. 5 A), suggesting that elevated Bmf gene expression in Bε, but not in Bγ, cells may account for their differential sensitivity to IL-21–mediated apoptosis.

To investigate whether the Bmf expressed in Bε cells is functional in its proapoptotic activity, Bmf cDNA was isolated from Bε cells and used to prepare several mutants of enhanced GFP–fused Bmf. These mutations included an A69P mutation in the dynein light chain 2 binding motif and an L138A mutation in the BH3 domain. These Bmf mutants were transfected into Baf3 cells. Upon IL-3 deprivation, mock transfectants underwent apoptosis. Transfection with WT Bmf or Bmf-A69P to Baf3 cells also significantly augmented apoptosis (Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20062206/DC1). However, reduced apoptosis was seen in Baf3 cells transfected with BH3 mutants, such as Bmf-L138A or Bmf-A69P/L138A (Fig. S3), indicating that Bmf in Bε cells is functional and the BH3 domain of the protein is important for mediating its proapoptotic activity.

Based on the understanding of proapoptotic activity of Bmf expressed in Bε cells, we investigated the formation of Bmf–Bcl-2 complexes in Bε cells after activation with IL-21. Bmf in Bε cells faintly binds to Bcl-2 in unstimulated cells (Fig. 5 B, left). However, when Bε cells were stimulated with IL-21, the formation of Bmf–Bcl-2 complexes was significantly augmented (Fig. 5 B, right).

BCG-mediated IL-21 induction in human Vα24 NKT cells

To determine how widespread our findings are, we investigated whether IL-21 and Vα24 NKT cells are required for the BCG-mediated suppression of human IgE responses. When human PBMCs were stimulated with α-GalCer or BCG, a significant up-regulation of IL-21 mRNA was detected by quantitative PCR (Fig. 6 A). The BCG-induced up-regulation of IL-21 mRNA was effectively suppressed by blocking with antibodies against CD1d, IL-12p40/p70, or both (Fig. 6 B), indicating that the CD1d-restricted NKT cell–dependent suppression of IgE responses observed in mice also operates in the human immune system. IL-21 mRNA expression by anti-CD1d and anti–IL-12 treatment was significantly reduced but was not as effective as in the mouse Vα14 NKT cell system (Fig. 3 D), perhaps suggesting a significant contribution of human conventional $CD4^+$ T cells (Fig. S2 B).

To evaluate in vivo responses, we inoculated BCG into healthy volunteers and examined IL-21 mRNA levels in PBMCs 1 wk later. There was a significant up-regulation of IL-21 mRNA levels in five out of six individuals (Fig. 6 C), and, furthermore, IL-21 suppressed IgE production by human Bε cells (Fig. 6 D, left). As expected, the addition of BCG-stimulated, but not control, PBMCs significantly inhibited IgE production (Fig. 6 D, right).

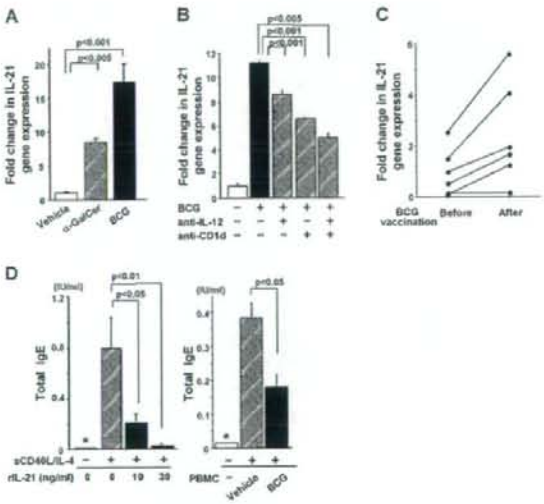

Figure 6. IL-21 mRNA expression and IL-21–induced IgE suppression in humans. (A) IL-21 mRNA expression in PBMCs. 10^6 human PBMCs were stimulated with 100 ng/ml α-GalCer or 50 μg/ml BCG and examined for IL-21 expression by quantitative real-time PCR with Taqman probes. The data are representative of five donors. (B) IL-12 and CD1d are required for IL-21 expression. 10^6 PBMCs were stimulated in vitro with 50 μg/ml BCG in the presence of 10 μg/ml anti-CD1d and/or anti–IL-12p40/p70 mAb. Representative data from five donors are shown. (C) IL-21 mRNA expression in PBMCs. Healthy volunteers were inoculated intradermally with BCG (two drops of 26.7 mg/ml of BCG emulsion per person). In A–C, the data for IL-21 expression were normalized to 18S ribosomal RNA expression, and relative expression levels are shown. Statistical analysis was performed using a matched pairs *t* test in C. (D) Suppression of IgE production. Left, suppression of IgE production by IL-21. 2×10^5 human B cells were cultured with sCD40L and IL-4 in the presence of human IL-21 for 14 d. Right, suppression of IgE production by BCG-activated human PBMCs. 10^5 Bε cells were co-cultured with 10^5 PBMCs, sCD40L, and IL-4 in the presence of 50 μg/ml BCG for 14 d. Total IgE was measured by ELISA. Values are expressed as mean ± SD of triplicate cultures. The asterisks (*) indicate that the IgE levels are below the detection limit for total IgE (<0.014 IU/ml). Data shown are representative of three donors. Results were expressed as a fold difference in human IL-21 gene expression relative to a control sample (vehicle) after being normalized with 18S ribosomal RNA expressions in each sample.

Published December 18, 2006

DISCUSSION

It is widely accepted that the mechanistic basis of the hygiene hypothesis for suppression of IgE responses is an increase in the Th1/Th2 ratio (12). However, in reality, the Th1 response exacerbates allergic reactions, as human asthma is associated with the production of IFN-γ, a cytokine that appears to contribute to the pathogenesis of the disease (35). Furthermore, the adoptive transfer of allergen-specific Th1 cells causes severe airway inflammation (36). Thus, a shift in the Th1/Th2 ratio alone cannot explain all of the immunological findings observed in allergic diseases (1). Furthermore, there are several studies suggesting that BCG vaccination has little or no effect on the development and prevalence of allergic diseases (37, 38). Therefore, it is necessary to better understand the precise mechanism of IgE suppression in BCG-treated animals or humans.

In this study, neither a Th1/Th2 imbalance nor an involvement of regulatory T cells was observed in response to BCG treatment (Fig. 1). Instead, we demonstrated that IL-21–induced Bε cell apoptosis is the mechanism responsible for BCG-mediated suppression of IgE production (Figs. 1, 3, and 5). Because the human IL-21 responses to BCG vaccination were heterogeneous (Fig. 6 C), it seems likely that the magnitude of the response in each individual could cause different degrees of BCG-induced IgE suppression and might be prognostic.

Previous studies have indicated that IL-21 is preferentially expressed by activated $CD4^+$ T cells (20), the results that are partially in agreement with the present data, as half of peripheral Vα14 NKT cells are $CD4^+$ (39, 40). Interestingly, upon anti-CD3 mAb stimulation, Vα14 NKT cells, but not conventional T cells, preferentially expressed IL-21 (Fig. S2 A), similar to the results with BCG (Fig. 3 B). Therefore, the major IL-21 producers in response to BCG in mice are Vα14 NKT cells.

It has been proposed that, for full activation of Vα14 NKT cells to produce IFN-γ, two signals are required: one CD1d-dependent and the other TLR-mediated IL-12–dependent signals (31). In agreement with this, IL-21 expression by BCG-activated Vα14 NKT cells was significantly inhibited by blocking with antibodies to IL-12 and/or CD1d (Fig. 3 D). Therefore, it is likely that Vα14 NKT cells recognize endogenous antigens presented by CD1d molecules but require IL-12 signals to produce IL-21. Nevertheless, it is still possible that glycolipid BCG components such as phosphatidylinositol mannoside may directly stimulate Vα14 NKT cells to produce IL-21 in a CD1d-dependent manner (41, 42).

In terms of the receptors on DCs that are required for BCG recognition and signal transduction, we showed in this study that BCG-induced IL-12 production is IRAK-4 and MyD88 dependent (Fig. 2 E and Fig. S1). These results in mice are consistent with a recent report indicating that BCG cannot induce IL-12 or IFN-γ production by PBMCs from IRAK-4–deficient patients (43). In addition, it has been reported that BCG enhances NF-κB–dependent gene transcription through the activation of phosphatidylinositol 3 kinase and c-Jun N-terminal kinase cascades (44). The activated NF-κB is then liberated for nuclear translocation and transactivates a variety of immune response genes, including IL-12.

In contrast to a previous report that implicated TLR2 and TLR4 in the recognition of mycobacterial antigens (32), we could not identify any involvement of these receptors in IL-12 production by BCG-stimulated BM-DCs (Fig. 2, C and D). In agreement with our findings, it has recently been reported that TLR2/4 double KO mice infected with live BCG have normal adaptive immune responses and survived as long as WT mice (45). As whole BCG contains multiple components including mycobacterial glycolipids, proteins, and DNA, several receptors that use IRAK-4 and MyD88 as the signal transducer appear to be involved in the complex recognition of BCG.

In IL-21R–deficient mice, the level of circulating IgE is high, whereas that of IgG1 is low (23, 46). Similarly, in human B cells, IL-21 inhibits IgE production and stimulates IgG4 (analogous to mouse IgG1) production (19). These results suggest that IL-21 differentially regulates IgE and IgG1 (IgG4 in humans) class switching. In fact, Suto et al. (18) reported that IL-21 specifically suppresses IgE production by inhibiting germ line Cε transcripts. Our present findings do not exclude this possibility. IL-21 has also been reported to induce apoptosis in resting and activated B cells by reducing the expression levels of apoptosis-related genes (25, 26). However, in this report, we have shown that IL-21 selectively induces apoptosis in Bε, but not Bγ, cells (Fig. 4 D). Thus, our findings that BCG-activated IL-21–expressing Vα14 NKT cells suppressed IgE production even after class switching (Fig. 4 C) suggests that the role of IL-21 on Bε cells is to control cell growth and viability, rather than to regulate the differentiation and maturation of these cells.

We found that expression of a proapoptotic gene, Bmf, was significantly higher in Bε cells than in Bγ cells (Fig. 5 A). Under physiological conditions, Bmf, which is a BH3 domain–only Bcl-2 family member that inhibits Bcl-2 function and accelerates apoptosis, binds to myosin V motors via the dynein light chain 2 domain of Bmf (34). In response to certain cellular damage signals, Bmf is supposed to be released from the myosin V motors and trigger apoptosis (34). Because Bmf from Bε cells induced apoptosis and a mutation in the BH3 domain of Bmf failed to induce apoptosis (Fig. S3), we confirmed that Bmf expressed in Bε cells is functional, and that the BH3 domain is important for the binding to Bcl-2 and is essential for its proapoptotic activity. In fact, the binding of Bmf with Bcl-2 was up-regulated by IL-21R signaling (Fig. 5 C). Therefore, BCG-mediated Bε cell apoptosis is due to the augmented formation of Bmf–Bcl-2 complexes generated by IL-21R signaling in Bε cells.

Finally, we defined the mechanism of BCG-induced IL-21–dependent suppression of IgE production in humans (Fig. 6). In a broader context, these findings may explain the mechanisms underlying the BCG-mediated suppression of allergic diseases and the epidemiological data indicating a reduction in the morbidity of allergic diseases in patients who

Published December 18, 2006

have been infected with *Mycobacterium tuberculosis*. Interestingly, IL-21–mediated B cell responses in C57BL/6 mice differ from those in BALB/c mice (26), suggesting that there is a genetic polymorphism with respect to the outcome of IL-21 signaling in B cells. In fact, a recent report indicated that polymorphisms in the IL-21R gene locus differentially affect serum IgE levels in humans (47). In this study, consistent with our data, the levels of IL-21 expression induced by BCG stimulation varied among the individuals examined (Fig. 6 C). These results suggest that the response to BCG in humans is dependent, at least in part, on genetic background. The specific genes responsible for the heterogeneity in BCG-mediated IL-21 production have not been identified. However, this observation may be applied to the development of diagnostic or therapeutic strategies in which the levels of IL-21 expression are used to evaluate the efficacy of BCG treatment, or in determining the potential benefit of therapy using bacterial products such as CpG for allergic diseases.

MATERIALS AND METHODS

Mice. 7–10-wk-old female BALB/c mice were purchased from Japan CREA Inc. Vα14 NKT-deficient (Vα14 NKT KO) mice on a BALB/c background (48), IRAK-4 KO (49), TLR2 KO, TLR4 KO, and MyD88 KO mice (50, 51) have been described. TLR2 and TLR4 double KO mice were generated by breeding. Mice were kept under specific pathogen-free conditions, maintained on an OVA-free diet, and treated in accordance with the guidelines for animal care at RIKEN Research Center for Allergy and Immunology.

Allergic sensitization and BCG. Allergic epicutaneous sensitization was performed as described previously (27). In brief, a 1-cm^2 sterile patch infused with 100 μl of PBS solution with or without 100 μg OVA (grade V; Sigma-Aldrich) was placed on the shaved back of mice and fixed in place with a bio-occlusive dressing and an elastic bandage. Patches were left on for 48 h and removed. The sensitization course was repeated at the same skin site every week for 4 wk. For BCG vaccination, mice were given a weekly i.p. injection of BCG (500 μg/mouse) or PBS at the time of OVA sensitization. The attenuated BCG (strain Tokyo) was purchased from the Japan BCG Laboratory.

Flow cytometry. Cells were stained with antibodies after adding 2.4G2 (BD Biosciences) for Fc blocking. The following antibodies were used: FITC–anti-CD19 (1D3), FITC–anti-IgE (R35-72), APC–anti-IgG1 (X59), FITC–anti-TCRβ (H57-597), APC–anti–IL-12p40/70 (C15.6), and PE–anti-CD11c (HL3; BD Biosciences). PE-conjugated α-GalCer–loaded CD1d tetramer (α-GalCer/CD1d tetramer) was prepared as described previously (52). For intracellular staining, BM-DCs were fixed and permeabilized with BD Cytofix and Cytoperm kits after staining with PE–anti-CD11c. They were then stained with APC–anti–IL-12p40/70. FACS analysis of at least 10,000 cells and cell sorting were performed with a FACSCalibur (BD Biosciences) with FlowJo software (TreeStar) or with a MoFlo cell sorter (DakoCytomation).

Cell preparations and cultures. 2×10^6 BM-DCs obtained by culturing BM for 6 d with 10 ng/ml GM-CSF were further cultured in the presence or absence of BCG, CpG, LPS (Invivogen), PGN from *Escherichia coli* (Invivogen), or 10 μg/ml anti-CD3 mAb (2C11; BD Biosciences) for 48 h at 37°C. For blocking experiments, mAb against CD1d or IL-12p40/p70 (clones 1B1 and C17.8, respectively; BD Biosciences), or an isotype control was added at a concentration of 20 μg/ml after 2.4G2 treatment. TCRβ$^+$ cells or Vα14 NKT cells with a purity of >98% were obtained from liver MNCs (52) using an Auto MACS (Miltenyi Biotec) after staining with FITC–anti-TCRβ and sorting with anti-FITC magnetic beads (Miltenyi Biotec). Vα14 NKT cells were then isolated from TCRβ$^+$ cells by MoFlo using PE–α-GalCer/CD1d tetramer. Conventional T or CD4$^+$ T cells were isolated from an α-GalCer/CD1d tetramer$^-$ fraction of TCRβ$^+$ liver MNCs. Bε and Bγ cells generated from splenic CD19$^+$ cells in the presence of 10 μg/ml sCD40L (ALX-850-075; Qbiogene) and 20 ng/ml of recombinant IL-4 (PeproTech) for 3 d (33) were cultured for 30 h for the apoptosis assay or for an additional 5 d to investigate IgE responses.

ELISA. Cytokines (IL-12p70 and IL-6) and Ig subclasses (IgG1, IgG2a, and IgE) were measured by ELISA using kits or sets of antibodies (BD Biosciences) according to the manufacturer's protocol. Specific antibodies were also measured as described previously (7).

RT-PCR. Total RNA was extracted by RNAeasy (QIAGEN), and cDNA was synthesized with random primers after DNase treatment. The following RT-PCR primer sets were used for mouse genes: IL-21, 5′-CCCTTGTCTGTCTGGTAGTCATC-3′ and 5′-ATCACAGGAAGGGCATTTAGC-3′; IgE (Cε), 5′-AGGAACCCTCAGCTCTACCC-3′ and 5′-GCCAGCTGACAGAGACATCA-3′; mIL-21R, 5′-TGTCAATGTGACGGACCAGT-3′ and 5′-CAGCATAGGGGTCTCTGAGG-3′; γc, 5′-GTCGACAGAGCAAGCACCATGTTGAAACTA-3′ and 5′-GGATCCTGGGATCACAAGATTCTGTAGGTT-3′; Bmf, 5′-CAGACCCTCAGTCCAGCTTC-3′ and 5′-CGTATGAAGCCGATGGAACT-3′; Bcl-2, 5′-GGTGGTGGAGGAACTCTTCA-3′ and 5′-CATGCTGGGGCCATATAGTT-3′; and HPRT, 5′-AGCGTCGTGATTAGCGATG-3′ and 5′-CTTTTATGTCCCCCGTTGAC-3′. The numbers of PCR cycles were as follows: 30 for HPRT; 35 for IgE, γc, and IL-21R; 40 for IL-21 and Bmf; and 45 for Bcl-2. The amounts of cDNA were standardized by quantification of the housekeeping gene HPRT using primers for mouse samples. The human IL-21 mRNA levels were quantified by real-time quantitative PCR on the ABI Prism 7000 sequence detection system (Applied Biosystems) by using TaqMan assay kits and TaqMan Gene Expression Assays (primers and TaqMan probes).

Electrophoretic mobility shift assay. 2×10^6 BM-DCs were stimulated with 50 μg/ml BCG or 1.0 μM CpG-B for the indicated periods. Nuclear extracts were prepared and used for Gel Shift Assay Systems (Promega) as described previously (50).

Bε cell–derived Bmf and its mutants. cDNAs encoding bmf were amplified from Bε cells by PCR using primers 5′-CCGAATTCGGATGGAGCCACCTCAGTGTGT-3′ and 5′-GCGGCCGCCTGCATTCCTGGTGATCCAT-3′ (EcoRI and NotI sites for cloning are underlined). The amplified products were cloned using the pGEM-T Easy Vector System (Promega). Mutant cDNAs were generated by PCR using point-mutated primer pairs.

Immunoprecipitation and Western blotting. Interaction of Bmf with Bcl-2 in Bε cells was detected by immunoprecipitation with anti–Bcl-2 mAb (clone 7; BD Transduction Laboratories) and subsequent immunoblotting with anti-Bmf rabbit antibody (Cell Signaling). The protein levels were visualized by ECL (GE Healthcare) using horseradish peroxidase–conjugated Protein A/G (Pierce Chemical Co.).

Human studies. All human specimens were obtained under informed consent. The protocol for the human research project has been approved by the Ethics Committee of Chiba University and RIKEN, and conformed to the provisions of the Declaration of Helsinki in 1995. 10^8 PBMCs from healthy volunteers were prepared by Ficoll-Paque density gradient centrifugation and used for the cultures. Human recombinant IL-21 was purchased from BIOSOURCE Inc. Human total IgE was measured with a sensitive immune assay (GE Healthcare).

Statistical analysis. Statistical analyses were performed using the Student's *t* test or matched pairs *t* test. $P < 0.05$ was considered statistically significant.

Online supplemental material. Fig. S1 provides data demonstrating that MyD88 signaling in DCs is required for BCG-induced activation. Fig. S2 contains data demonstrating IL-21 mRNA expression by NKT cells, $CD4^+$ T cells, and $CD8^+$ T cells of murine and human origin. Fig. S3 provides the data indicating proapoptotic activity of Bα cell–derived Bmf and functional domain analysis using mutant Bmf in Baf3 cells. The online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20062206/DC1.

The authors thank Prof. Peter Burrows for critical reading and Ms. Norie Takeuchi for secretarial assistance.

This work was partly supported by a grant from The Ministry of Education, Culture, Sports, Science and Technology (RR2002).

The authors have no conflicting financial interests.

Submitted: 13 October 2006
Accepted: 21 November 2006

REFERENCES

1. Umetsu, D.T., J.J. Mcintire, O. Akbari, C. Macaubas, and R.H. Dekruyff. 2002. Asthma: an epidemic of dysregulated immunity. *Nat. Immunol.* 3:715–720.
2. Cookson, W.O., and M.F. Moffatt. 1997. Asthma: an epidemic in the absence of infection? *Science.* 275:41–42.
3. Von Mutius, E. 2000. The environmental predictors of allergic disease. *J. Allergy Clin. Immunol.* 105:9–19.
4. Shaheen, S.O., P. Aaby, A.J. Hall, D.J. Barker, C.B. Heyes, A.W. Shiell, and A. Goudiaby. 1996. Measles and atopy in Guinea-Bissau. *Lancet.* 347:1792–1796.
5. Shirakawa, T., T. Enomoto, S. Shimazu, and J.M. Hopkin. 1997. The inverse association between tuberculin responses and atopic disorder. *Science.* 275:77–79.
6. Adams, J.F., E.H. Scholvinck, R.P. Gie, P.C. Potter, N. Beyers, and A.D. Beyers. 1999. Decline in total serum IgE after treatment for tuberculosis. *Lancet.* 353:2030–2033.
7. Herz, U., K. Gerhold, C. Gruber, A. Braun, U. Wahn, H. Renz, and K. Paul. 1998. BCG infection suppresses allergic sensitization and development of increased airway reactivity in an animal model. *J. Allergy Clin. Immunol.* 102:867–874.
8. Wang, C.C., and G.A. Rook. 1998. Inhibition of an established allergic response to ovalbumin in BALB/c mice by killed Mycobacterium vaccae. *Immunology.* 93:307–313.
9. Yang, X., S. Wang, Y. Fan, and L. Zhu. 1999. Systemic mycobacterial infection inhibits antigen-specific immunoglobulin E production, bronchial mucus production and eosinophilic inflammation induced by allergen. *Immunology.* 98:329–337.
10. Cavallo, G.P., M. Elia, D. Giordano, C. Baldi, and R. Cammarota. 2002. Decrease of specific and total IgE levels in allergic patients after BCG vaccination: preliminary report. *Arch. Otolaryngol. Head Neck Surg.* 128:1058–1060.
11. Krieg, A.M. 2002. CpG motifs in bacterial DNA and their immune effects. *Annu. Rev. Immunol.* 20:709–760.
12. Renz, H., and U. Herz. 2002. The bidirectional capacity of bacterial antigens to modulate allergy and asthma. *Eur. Respir. J.* 19:158–171.
13. Akbari, O., P. Stock, R.H. Dekruyff, and D.T. Umetsu. 2003. Role of regulatory T cells in allergy and asthma. *Curr. Opin. Immunol.* 15:627–633.
14. Stassen, M., H. Jonuleit, C. Muller, M. Klein, C. Richter, T. Bopp, S. Schmitt, and E. Schmitt. 2004. Differential regulatory capacity of CD25+ T regulatory cells and preactivated CD25+ T regulatory cells on development, functional activation, and proliferation of Th2 cells. *J. Immunol.* 173:267–274.
15. Robinson, D.S., M. Larche, and S.R. Durham. 2004. Tregs and allergic disease. *J. Clin. Invest.* 114:1389–1397.
16. Taniguchi, M., M. Harada, S. Kojo, T. Nakayama, and H. Wakao. 2003. The regulatory role of Valpha14 NKT cells in innate and acquired immune response. *Annu. Rev. Immunol.* 21:483–513.
17. Cui, J., N. Watanabe, T. Kawano, M. Yamashita, T. Kamata, C. Shimizu, M. Kimura, E. Shimizu, J. Koike, H. Koseki, et al. 1999. Inhibition of T helper cell type 2 cell differentiation and immunoglobulin E response by ligand-activated Vα14 natural killer T cells. *J. Exp. Med.* 190:783–792.
18. Suto, A., H. Nakajima, K. Hirose, K. Suzuki, S. Kagami, Y. Seto, A. Hoshimoto, Y. Saito, D.C. Foster, and I. Iwamoto. 2002. Interleukin 21 prevents antigen-induced IgE production by inhibiting germ line C(epsilon) transcription of IL-4-stimulated B cells. *Blood.* 100:4565–4573.
19. Wood, N., K. Bourque, D.D. Donaldson, M. Collins, D. Vercelli, S.J. Goldman, and M.T. Kasaian. 2004. IL-21 effects on human IgE production in response to IL-4 or IL-13. *Cell. Immunol.* 231:133–145.
20. Parrish-Novak, J., S.R. Dillon, A. Nelson, A. Hammond, C. Sprecher, J.A. Gross, J. Johnston, K. Madden, W. Xu, J. West, et al. 2000. Interleukin 21 and its receptor are involved in NK cell expansion and regulation of lymphocyte function. *Nature.* 408:57–63.
21. Parrish-Novak, J., D.C. Foster, R.D. Holly, and C.H. Clegg. 2002. Interleukin-21 and the IL-21 receptor: novel effectors of NK and T cell responses. *J. Leukoc. Biol.* 72:856–863.
22. Habib, T., A. Nelson, and K. Kaushansky. 2003. IL-21: a novel IL-2-family lymphokine that modulates B, T, and natural killer cell responses. *J. Allergy Clin. Immunol.* 112:1033–1045.
23. Ozaki, K., R. Spolski, C.G. Feng, C.F. Qi, J. Cheng, A. Sher, H.C. Morse III, C. Liu, P.L. Schwartzberg, and W.J. Leonard. 2002. A critical role for IL-21 in regulating immunoglobulin production. *Science.* 298:1630–1634.
24. Purkerson, J.M., and P.C. Isakson. 1994. Independent regulation of DNA recombination and immunoglobulin (Ig) secretion during isotype switching to IgG1 and IgE. *J. Exp. Med.* 179:1877–1883.
25. Mehta, D.S., A.L. Wurster, M.J. Whitters, D.A. Young, M. Collins, and M.J. Grusby. 2003. IL-21 induces the apoptosis of resting and activated primary B cells. *J. Immunol.* 170:4111–4118.
26. Jin, H., R. Carrio, A. Yu, and T.R. Malek. 2004. Distinct activation signals determine whether IL-21 induces B cell costimulation, growth arrest, or Bim-dependent apoptosis. *J. Immunol.* 173:657–665.
27. Nelde, A., M. Teufel, C. Hahn, A. Duschl, W. Sebald, E.B. Brocker, and S.M. Grunewald. 2001. The impact of the route and frequency of antigen exposure on the IgE response in allergy. *Int. Arch. Allergy Immunol.* 124:461–469.
28. Gould, H.J., B.J. Sutton, A.J. Beavil, R.L. Beavil, N. Mccloskey, H.A. Coker, D. Fear, and L. Smurthwaite. 2003. The biology of IgE and the basis of allergic disease. *Annu. Rev. Immunol.* 21:579–628.
29. Hayakawa, Y., K. Takeda, H. Yagita, L. Van Kaer, I. Saiki, and K. Okumura. 2001. Differential regulation of Th1 and Th2 functions of NKT cells by CD28 and CD40 costimulatory pathways. *J. Immunol.* 166:6012–6018.
30. Yoshimoto, T., A. Bendelac, C. Watson, J. Hu-Li, and W.E. Paul. 1995. Role of NK1.1+ T cells in a TH2 response and in immunoglobulin E production. *Science.* 270:1845–1847.
31. Brigl, M., L. Bry, S.C. Kent, J.E. Gumperz, and M.B. Brenner. 2003. Mechanism of CD1d-restricted natural killer T cell activation during microbial infection. *Nat. Immunol.* 4:1230–1237.
32. Tsuji, S., M. Matsumoto, O. Takeuchi, S. Akira, I. Azuma, A. Hayashi, K. Toyoshima, and T. Seya. 2000. Maturation of human dendritic cells by cell wall skeleton of Mycobacterium bovis bacillus Calmette-Guerin: involvement of toll-like receptors. *Infect. Immun.* 68:6883–6890.
33. Snapper, C.M., P. Zelazowski, F.R. Rosas, M.R. Kehry, M. Tian, D. Baltimore, and W.C. Sha. 1996. B cells from p50/NF-kappa B knockout mice have selective defects in proliferation, differentiation, germ-line CH transcription, and Ig class switching. *J. Immunol.* 156: 183–191.
34. Puthalakath, H., A. Villunger, L.A. O'Reilly, J.G. Beaumont, L. Coultas, R.E. Cheney, D.C. Huang, and A. Strasser. 2001. Bmf: a proapoptotic BH3-only protein regulated by interaction with the myosin V actin motor complex, activated by anoikis. *Science.* 293:1829–1832.
35. Corrigan, C.J., and A.B. Kay. 1990. CD4 T-lymphocyte activation in acute severe asthma. Relationship to disease severity and atopic status. *Am. Rev. Respir. Dis.* 141:970–977.
36. Hansen, G., G. Berry, R.H. Dekruyff, and D.T. Umetsu. 1999. Allergen-specific Th1 cells fail to counterbalance Th2 cell-induced

Published December 18, 2006

airway hyperreactivity but cause severe airway inflammation. *J. Clin. Invest.* 103:175–183.

37. Arikan, C., N.N. Bahceciler, G. Deniz, M. Akdis, T. Akkoc, C.A. Akdis, and I.B. Barlan. 2004. Bacillus Calmette-Guerin-induced interleukin-12 did not additionally improve clinical and immunologic parameters in asthmatic children treated with sublingual immunotherapy. *Clin. Exp. Allergy.* 34:398–405.
38. Vargas, M.H., D.A. Bernal-Alcantara, M.A. Vaca, F. Franco-Marina, and R. Lascurain. 2004. Effect of BCG vaccination in asthmatic schoolchildren. *Pediatr. Allergy Immunol.* 15:415–420.
39. Matsuda, J.L., O.V. Naidenko, L. Gapin, T. Nakayama, M. Taniguchi, C.R. Wang, Y. Koezuka, and M. Kronenberg. 2000. Tracking the response of natural killer T cells to a glycolipid antigen using CD1d tetramers. *J. Exp. Med.* 192:741–754.
40. Benlagha, K., A. Weiss, A. Beavis, L. Teyton, and A. Bendelac. 2000. In vivo identification of glycolipid antigen-specific T cells using fluorescent CD1d tetramers. *J. Exp. Med.* 191:1895–1903.
41. Apostolou, I., Y. Takahama, C. Belmant, T. Kawano, M. Huerre, G. Marchal, J. Cui, M. Taniguchi, H. Nakauchi, J.J. Fournie, et al. 1999. Murine natural killer T(NKT) cells [correction of natural killer cells] contribute to the granulomatous reaction caused by mycobacterial cell walls. *Proc. Natl. Acad. Sci. USA.* 96:5141–5146.
42. Fischer, K., E. Scotet, M. Niemeyer, H. Koebernick, J. Zerrahn, S. Maillet, R. Hurwitz, M. Kursar, M. Bonneville, S.H. Kaufmann, and U.E. Schaible. 2004. Mycobacterial phosphatidylinositol mannoside is a natural antigen for CD1d-restricted T cells. *Proc. Natl. Acad. Sci. USA.* 101:10685–10690.
43. Feinberg, J., C. Fieschi, R. Doffinger, M. Feinberg, T. Leclerc, S. Boisson-Dupuis, C. Picard, J. Bustamante, A. Chapgier, O. Filipe-Santos, et al. 2004. Bacillus Calmette Guerin triggers the IL-12/IFN-gamma axis by an IRAK-4- and NEMO-dependent, non-cognate interaction between monocytes, NK, and T lymphocytes. *Eur. J. Immunol.* 34:3276–3284.
44. Darieva, Z., E.B. Lasunskaia, M.N. Campos, T.L. Kipnis, and W.D. Da Silva. 2004. Activation of phosphatidylinositol 3-kinase and c-Jun-N-terminal kinase cascades enhances NF-kappaB-dependent gene transcription in BCG-stimulated macrophages through promotion of p65/p300 binding. *J. Leukoc. Biol.* 75:689–697.
45. Nicolle, D., C. Fremond, X. Pichon, A. Bouchot, I. Maillet, B. Ryffel, and V.J. Quesniaux. 2004. Long-term control of Mycobacterium bovis BCG infection in the absence of Toll-like receptors (TLRs): investigation of TLR2-, TLR6-, or TLR2-TLR4-deficient mice. *Infect. Immun.* 72:6994–7004.
46. Kasaian, M.T., M.J. Whitters, L.L. Carter, L.D. Lowe, J.M. Jussif, B. Deng, K.A. Johnson, J.S. Witek, M. Senices, R.F. Konz, et al. 2002. IL-21 limits NK cell responses and promotes antigen-specific T cell activation: a mediator of the transition from innate to adaptive immunity. *Immunity.* 16:559–569.
47. Hecker, M., A. Bohnert, I.R. Konig, G. Bein, and H. Hackstein. 2003. Novel genetic variation of human interleukin-21 receptor is associated with elevated IgE levels in females. *Genes Immun.* 4:228–233.
48. Cui, J., T. Shin, T. Kawano, H. Sato, E. Kondo, I. Toura, Y. Kaneko, H. Koseki, M. Kanno, and M. Taniguchi. 1997. Requirement for Valpha14 NKT cells in IL-12-mediated rejection of tumors. *Science.* 278:1623–1626.
49. Suzuki, N., S. Suzuki, G.S. Duncan, D.G. Millar, T. Wada, C. Mirtsos, H. Takada, A. Wakeham, A. Itie, S. Li, et al. 2002. Severe impairment of interleukin-1 and Toll-like receptor signalling in mice lacking IRAK-4. *Nature.* 416:750–756.
50. Takeuchi, O., K. Hoshino, T. Kawai, H. Sanjo, H. Takada, T. Ogawa, K. Takeda, and S. Akira. 1999. Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity.* 11:443–451.
51. Adachi, O., T. Kawai, K. Takeda, M. Matsumoto, H. Tsutsui, M. Sakagami, K. Nakanishi, and S. Akira. 1998. Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. *Immunity.* 9:143–150.
52. Harada, M., K. Seino, H. Wakao, S. Sakata, Y. Ishizuka, T. Ito, S. Kojo, T. Nakayama, and M. Taniguchi. 2004. Down-regulation of the invariant Valpha14 antigen receptor in NKT cells upon activation. *Int. Immunol.* 16:241–247.

Allergology International. 2006;55:379-386

ORIGINAL ARTICLE

Omalizumab is Effective and Safe in the Treatment of Japanese Cedar Pollen-induced Seasonal Allergic Rhinitis

Kimihiro Okubo[1], Satoshi Ogino[2], Toshikazu Nagakura[3] and Takeru Ishikawa[4]

ABSTRACT

Background: Seasonal allergic rhinitis (SAR) induced by Japanese cedar pollen is a substantial problem in Japan. Omalizumab, a novel humanized monoclonal anti-immunoglobulin E (IgE) antibody, has already been proven to reduce symptoms associated with SAR. We investigated the safety and efficacy of omalizumab in the treatment of patients with Japanese cedar pollen-induced SAR compared to placebo.

Methods: A randomized, placebo-controlled, double-blind study was conducted in 100 Japanese patients with a history of moderate-to-severe SAR induced by Japanese cedar pollens. Omalizumab (150, 225, 300, or 375 mg) or placebo was administered subcutaneously every 2 or 4 weeks based on serum total IgE and body weight at baseline. The primary efficacy variable was the mean of daily nasal symptom medication scores (sum of the daily nasal symptom severity score and daily nasal rescue medication score) during the treatment period. Secondary efficacy variables included the daily ocular symptom medication score and related variables.

Results: Primary and all secondary efficacy variable scores were significantly lower in the omalizumab group than in the placebo group ($P < .01$). Serum free IgE levels markedly decreased in the omalizumab group and were associated with clinical efficacy. The overall incidence of injection site reactions was higher in the omalizumab group than in the placebo group; however, the adverse reaction profile was similar between the two groups when excluding injection site reactions. No anti-omalizumab antibodies were detected.

Conclusions: Omalizumab was effective and safe in the treatment of SAR induced by Japanese cedar pollen.

KEY WORDS

anti-IgE antibody, IgE, omalizumab, pollinosis, seasonal allergic rhinitis (SAR)

INTRODUCTION

Allergic rhinitis, especially Japanese cedar pollen-induced seasonal allergic rhinitis (SAR), is a highly prevalent disease in Japan.[1-3] Twelve percent of the total land in Japan is covered by Japanese cedar. Approximately 20 million people in Japan, who account for about 17% of the population, experience this form of SAR.[3] During the pollen season (February to April), the majority of these patients undergo treatment, *i.e.*, pharmacotherapy with antihistamines and corticosteroids, specific immunotherapy, folk medicine, and protective measures, such as masks, glasses, caps, and coats, to reduce pollen inhalation or their adhesion to the body.[1] The total direct cost of medical treatments for Japanese cedar pollen-induced SAR is estimated to be at least 120 billion yen ($1 billion dollars) annually.[4] Furthermore, daily activities and quality of life are reduced during the pollen season due to rhinoconjunctival symptoms or pharmacological side-effects.[5] Thus, SAR induced by Japanese cedar pollen is a substantial social problem in Ja-

[1]The Department of Otorhinolaryngology, Nippon Medical School, [3]The Allergy and Internal Medicine, Yoga Clinic, Tokyo, [2]The Department of Health and Hygiene, Osaka University School of Medicine, Osaka and [4]Professor Emeritus, Kumamoto University School of Medicine, Kumamoto, Japan.
Correspondence: Kimihiro Okubo, MD, PhD, Department of Otorhinolaryngology, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113-8602, Japan.
Email: ent-kimi@nms.ac.jp
Received 9 December 2005. Accepted for publication 17 April 2006.

pan.

Omalizumab, a recombinant humanized monoclonal anti-IgE antibody, which binds to the serum free IgE molecule and forms small biologically inert complexes, blocks the interaction between IgE and effector cells which trigger the allergic response irrespective of allergen type.[6,7] Circulating free IgE can be reduced up to 99% with omalizumab,[8] thus suppressing the activation of effector cells (e.g., mast cells). Furthermore, an omalizumab-induced reduction in serum-free IgE levels eventually down-regulates FcεRI expression on basophils[8] and mast cells.[9] Down-regulation of the receptor reduces the availability of receptor sites for cross-linking of IgE. Based on this mechanism of action, omalizumab is expected to be effective for type I allergic diseases mediated by allergen-specific IgE antibodies.[10,11] Indeed, omalizumab has already been shown to be effective for birch- and ragweed-induced SAR, perennial allergic rhinitis (PAR), and allergic asthma,[12-19] and is now approved for the treatment of allergic asthma in the United States and Europe.

To investigate the safety and efficacy of omalizumab and to examine the appropriateness of its dose in Japanese patients with SAR, we conducted a randomized, placebo-controlled, double-blind study in Japanese patients with moderate-to-severe Japanese cedar pollen-induced SAR. This was the first clinical trial to treat Japanese SAR patients with omalizumab. On the basis of previous oversea studies, the dose and regimen which we employed in the present study were expected to reduce serumfree IgE levels to below 50 ng/mL, a level which is considered important to gain optimal efficacy.[20]

METHODS

STUDY SUBJECTS

Patients who met the following criteria were considered eligible for enrollment: age (20 to 64 years); a history of SAR induced by Japanese cedar pollen in at least 2 consecutive years; presentation of at least 4 of 8 moderate-to-severe symptoms (sneezing, itchy nose, runny nose, stuffy nose, itchy eyes, watery eyes, red eyes, and itchy throat), which persisted for one or more weeks during the last Japanese cedar pollen season; presence of IgE specific to Japanese cedar pollens (CAP-RAST: $\geq$2+) at baseline; serum total IgE levels of 30 to 700 IU/mL and body weights of 30 to 150 kg at baseline; and no symptoms of allergic rhinitis at 1 month prior to the onset of the screening period.

Patients who had a history of the following were excluded from the study: specific immunotherapy to Japanese cedar pollen in the previous 2 years; severe anaphylactoid or anaphylactic reactions; active or recent development (within 3 months) of any other type of rhinitis; positive reaction to omalizumab at screening; pregnant/nursing women; and serious medical conditions.

The present study was conducted in compliance with the current good clinical practice, and the protocol was approved by each institutional ethical committee. Prior to the onset of the study, written informed consent was obtained from all the patients who were enrolled.

STUDY DESIGN

This randomized, placebo-controlled, double-blind study was conducted in two regions of Japan (Tokyo and Osaka) between October 2001 and April 2002 and consisted of a 4-week screening period, a 12-week treatment period, and a 12-week follow-up period after final dosing. Following screening, eligible patients were assigned to receive omalizumab or placebo at a 1 : 1 ratio.

The start day of the Japanese cedar pollen scattering period was defined as the first of 2 consecutive days when $\geq$1 grain/cm^2 were counted; the final day of the pollen season was the first of 3 consecutive days when no grain was counted. The peak Japanese cedar pollen scattering period was defined as the span between the first and last days when $\geq$ 30 grains/cm^2 were counted.

DOSES AND ADMINISTRATION

Omalizumab (150, 225, 300, or 375 mg) or placebo was administered to patients subcutaneously every 2 or 4 weeks based on their serum total IgE level and body weight at baseline. The initial dose was administered at least at 1 month prior to the expected starting date of the Japanese cedar pollen scattering period. Omalizumab or placebo was administered to patients 3 or 6 times in total during the 12-week treatment period.

The following drugs were permitted as rescue medications: for nasal use [clemastine fumarate (tablet), sodium cromoglycate (nose drop), naphazoline nitrate (nose drop)] and for ocular use [sodium cromoglycate (eye drop)]. Concomitant use of agents were prohibited except for rescue medications. Specific immunotherapy was prohibited.

EVALUATION OF EFFICACY

Patients enrolled were requested to fill in the patient diary in order to describe their seven rhinoconjunctival symptoms (sneezing, itchy nose, runny nose, stuffy nose, itchy eyes, watery eyes, and red eyes) according to the 4-point scale (0: none, 1: mild, 2: moderate, and 3: severe) and to document rescue medication use, if any. Regarding each rescue medication, its usage was scored 1 point regardless of dose and frequency.

The primary efficacy variable was the mean of daily nasal symptom medication scores (DNSMS) during the treatment period. DNSMS (0–15 points) consisted of the sum of the daily nasal symptom severity

score (DNSS) (0–12 points) and the daily nasal rescue medication score (0–3 points).

Secondary efficacy variables included the daily ocular symptom medication score (DOSMS) (0–10 points) [sum of the daily ocular symptom severity score (DOSS) (0–9 points) and daily ocular rescue medication score (0–1 point)]; DNSS; the daily nasal rescue medication score; DOSS; the daily ocular rescue medication score; the consumption per day of rescue medications; and the proportion of days in which any rescue medication was taken.

ESTIMATION OF SERUM FREE IgE LEVELS

To investigate the relationship between serum IgE level and efficacy of omalizumab, serum free IgE levels were measured before dosing and at 4 and 12 weeks of the treatment period.[12]

EVALUATION OF SAFETY

Adverse events were examined throughout the treatment period. Laboratory tests and check-up of vital signs were conducted during the screening period and at 4 and 12 weeks of the treatment period.

During the screening period and at 12 weeks after final dosing, anti-omalizumab antibodies (IgG isotype) were measured using two solid-phase ELISA methods: one assay was to detect anti-omalizumab Fab responses; and another was to detect anti-omalizumab Fc responses.[12]

STATISTICAL ANALYSIS

One hundred patients, assigned to the omalizumab group and the placebo group at a 1 : 1 ratio, were required to detect a 0.30-point difference in the mean DNSMS between treatment groups. This calculation assumed 90% power at a significance level of 0.05, 2-sided, and at a standard deviation of 0.50 for the difference.

Regarding efficacy, the following hypothesis tests were used to examine study group comparability with respect to demographic and baseline characteristics: Fisher's exact test for gender; and Wilcoxon rank sum test for age, history of Japanese cedar pollen-induced SAR, IgE specific to Japanese cedar pollens, and serum total IgE at baseline.

The full analysis set was used to analyze the primary variable (*i.e.*, the mean DNSMS during the treatment period) and to analyze the mean DNSMS during the Japanese cedar pollen scattering period and the peak Japanese cedar pollen scattering period. These comparisons were based on the null hypothesis that there is no difference between the study groups. The mean DNSMS was analyzed using an ANCOVA model which included study group, location, and administration interval (2- or 4-week interval). The least-squares mean (LSM) for each group and the difference in LSM between the study groups were determined. The mean DOSMS, each of symptom severity scores, and each of rescue medication scores were also analyzed similarly to the analysis of the mean DNSMS.

The safety and tolerability of the study drugs are summarized by appropriate descriptive methods.

RESULTS

PATIENT CHARACTERISTICS

Ninety-eight of 100 randomized subjects received either of the study drugs: 48 received omalizumab (50 randomized) and 50 received placebo. The remaining two subjects in the omalizumab group withdrew during the screening period due to personal reasons. No significant difference was found between the omalizumab group and the placebo group with respect to patient characteristics (Table 1).

The Japanese cedar pollen scattering period started at the beginning of February and finished at the end of April (Fig. 1). All subjects received the first administration at least at 1 month prior to the starting date of the Japanese cedar pollen scattering period.

Five subjects (three receiving omalizumab and two receiving placebo) discontinued the study prematurely; among them, three (two receiving omalizumab and one receiving placebo) ceased the study due to adverse events.

EFFICACY

Daily Nasal Symptom Medication Score (DNSMS)

Changes in DNSMS over time are shown in Figure 1. DNSMS throughout the Japanese cedar pollen scattering period were consistently lower in the omalizumab group than in the placebo group.

The omalizumab group showed significantly lower mean DNSMS compared to the placebo group during the treatment period [LSM ± (SE), 1.391 ± 0.1769 for the omalizumab group and 2.499±0.1740 for the placebo group; $P < .001$; Figure 2A]. Statistical analyses revealed similar results with respect to the relevant scores during the Japanese cedar pollen scattering period (1.915 ± 0.2267 and 3.528 ± 0.2258, respectively; $P < .001$; Figure 2A) and the peak Japanese cedar pollen scattering period (2.586 ± 0.2907 and 4.511 ± 0.2886, respectively; $P<.001$; Figure 2A). During the Japanese cedar pollen scattering period, subjects with lower mean DNSMS were distributed predominantly in the omalizumab group than in the placebo group, with greater numbers of subjects with scores of 0–1 and >2–4 in the former and latter groups, respectively. About half of subjects in the omalizumab group had a mean DNSMS of ≤2 (mild or less severe symptoms) in contrast to 15% in the placebo group. More than 10% of subjects in the placebo group had scores of >6 (severe symptoms) compared to none in the omalizumab group (Fig. 2B).

As shown in Figure 1, the amount of Japanese cedar pollen in Tokyo was larger than that of Osaka

Table 1 Patient characteristics

	Omalizumab (n = 48)	Placebo (n = 50)
Gender		
Male	25	28
Age (years)		
Mean ± SD	32.2 ± 12.1	31.5 ± 12.3
Range	20-62	20-64
History of SAR induced by Japanese cedar pollens (years)		
Mean± SD	11.3 ± 6.2	9.6 ± 5.4
Range	4-35	3-26
Specific IgE levels against Japanese cedar pollens (CAP-RAST)*		
Class 2 (0.70-3.49 UA/mL)	3	0
Class 3 (3.50-17.49 UA/mL)	15	12
Class 4 (17.50-49.99 UA/mL)	19	25
Class 5 (50.00-99.99 UA/mL)	8	9
Class 6 (≥ 100 UA/mL)	3	4
Serum total IgE levels at baseline (IU/mL)		
Mean ± SD	193.7 ± 166.6	188.7 ± 145.8
Range	32.0 ± 590.0	34.0 ± 570.0

*: Specific IgE levels against Japanese cedar pollens at baseline were categorized into 7 groups (Classes 0 to 6), and a ≥2 class group was assessed to be positive against the allergen.

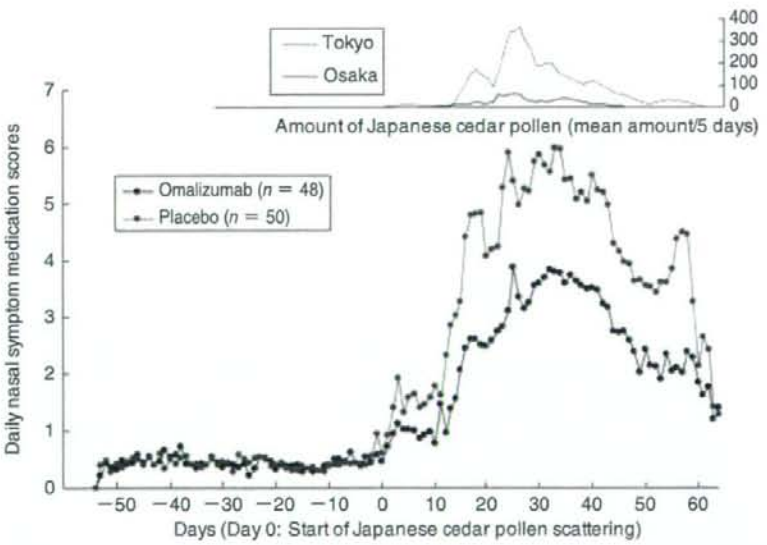

Fig. 1 Time-course changes in daily nasal symptom medication score (FAS) and in amount of Japanese cedar pollen. Day 0 represents the start day of the Japanese cedar pollen scattering period in Tokyo and Osaka.

(5648 grains/cm^2 for Tokyo and 913 grains/cm^2 for Osaka). Although in the placebo group as well as in the omalizumab group, the subgroup of Tokyo showed higher mean DNSMS compared to that of Osaka, the mean DNSMS were consistently lower in the omalizumab group than in the placebo group in Tokyo and Osaka, respectively [Mean ± (SE), 3.020 ± 0.2576 and 4.697 ± 0.3390 for Tokyo, 1.141 ± 0.2423 and 2.705 ± 0.2682 for Osaka, the Japanese cedar pollen scattering period]. Statistically, there was no interaction between the treatment group and the region (P = .8429).

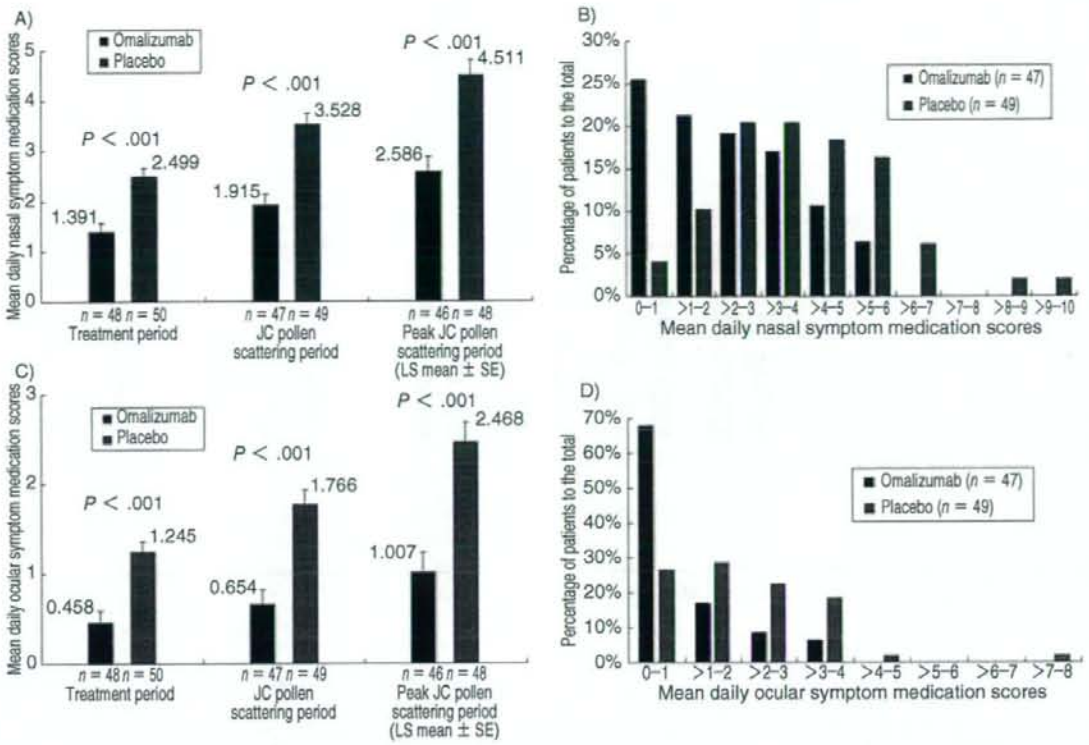

Fig. 2 **A**) Mean daily nasal symptom medication scores (DNSMS) and **C**) mean daily ocular symptom medication scores (DOSMS) during the treatment period, the Japanese cedar (JC) pollen scattering period, and the peak JC pollen scattering period. Percentages of the total of patients with **B**) mean DNSMS and **D**) mean DOSMS during the JC pollen scattering period.

Daily Nasal Rescue Medication Score

The mean daily nasal rescue medication scores were significantly lower in the omalizumab group than in the placebo group during the three evaluation periods (e.g., 0.055 ± 0.0503 and 0.260 ± 0.0499, respectively; $P = .002$, the peak Japanese cedar pollen scattering period).

Daily ocular symptom medication score (DOSMS)

The omalizumab group had significantly lower mean DOSMS compared to the placebo group during the treatment period (0.458 ± 0.1248 and 1.245 ± 0.1227, respectively; $P < .001$; Fig. 2C). Statistical analyses revealed similar results with respect to the relevant scores during the Japanese cedar pollen scattering period (0.654 ± 0.1675 and 1.766 ± 0.1688, respectively; $P < .001$) and the peak Japanese cedar pollen scattering period (1.007 ± 0.2244 and 2.468 ± 0.2228, respectively; $P<.001$). During the Japanese cedar pollen scattering period, approximately 70% (32/47) of subjects in the omalizumab group had ocular symptom medication scores of≤1 (Fig. 2D).

Daily Ocular Rescue Medication Score

The mean ocular rescue medication scores were significantly lower in the omalizumab group than in the placebo group during the three evaluation periods (e.g., 0.031 ± 0.0360 and 0.191 ± 0.0357, respectively; $P<.001$, the peak Japanese cedar pollen scattering period).

Daily nasal and ocular symptom severity scores (DNSS & DOSS)

The omalizumab group had significantly lower mean DNSS compared to the placebo group during the three evaluation periods (e.g., 1.880 ± 0.2183 and 3.349 ± 0.2175, respectively; $P < .001$, the Japanese cedar pollen scattering period). Each of the mean DNSS and DOSS during the three evaluation periods (sneezing, runny nose, stuffy nose, itchy nose, itchy eyes, watery eyes, and red eyes) was significantly lower in the omalizumab group (P values ranging from < .001 to .003; Fig. 3).

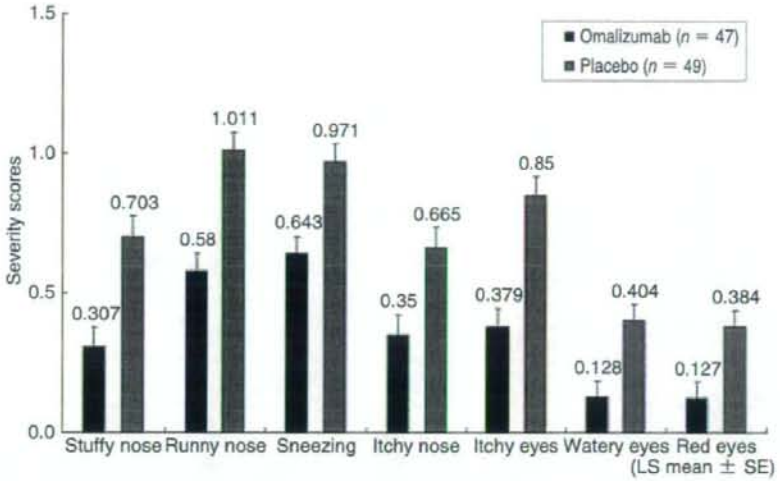

Fig. 3 Effects of omalizumab on each of the mean daily nasal and ocular symptom severity scores (FAS) during the Japanese cedar pollen scattering period ($P < .001$ for all variables). Statistically significant differences were noted during the treatment period and peak Japanese cedar pollen scattering periods.

Use of Rescue Medications

The mean consumption per day of each of the three rescue medications [clemastine fumarate (tablet), sodium cromoglycate (nose drop, eye drop)] was significantly lower in the omalizumab group than in the placebo group during the three evaluation periods (P values ranging from .002 to .017), and naphazoline nitrate (nose drop) tended to show a significant difference in consumption. The proportions of days in which any rescue medication was taken were almost 5-fold higher in the placebo group than in the omalizumab group (e.g., 25.4% and 5.6%, respectively; $P < .001$, the peak Japanese cedar pollen scattering period).

SERUM FREE IgE LEVELS

Serum free (total) IgE levels in the omalizumab group and the placebo group at baseline were at similar levels (Table 1). After administrations, serum free IgE levels in the omalizumab group decreased markedly, compared to the baseline levels to below 50 ng/mL at 4 and 12 weeks of the treatment period in all subjects (range from 6.1 ng/mL to 39.6 ng/mL). In the placebo group, serum free IgE levels were comparable to the baseline levels throughout the treatment period (range from 39.7 ng/mL to 1314 ng/mL).

SAFETY

Treatment with omalizumab was generally well tolerated. Due to the higher overall incidence of injection site reactions in the omalizumab group, the overall incidences of drug-related adverse events were significantly higher in the omalizumab group than in the placebo group; nevertheless, the adverse reaction profile was similar between the study groups when excluding injection site reactions (Table 2). One serious adverse event (colitis ulcerative) was reported in one subject in the omalizumab group, who was subsequently withdrawn from this study. However, the investigator considered its causality with the drug unlikely. Another subject in the omalizumab group and one subject in the placebo group discontinued treatment because of non-serious adverse events which were not drug-related. There were no anaphylactic reactions, and neither evidence of immune complex disease, nor clinically important abnormalities in vital signs and laboratory tests were found. No anti-omalizumab antibodies were detected.

DISCUSSION

This randomized, placebo-controlled, double-blind study revealed that omalizumab was generally well tolerated and was effective in preventing and controlling rhinoconjunctival symptoms associated with Japanese cedar pollen-induced SAR and in reducing rescue medication use for rhinoconjunctival symptoms.

Although the amount of Japanese cedar pollen in Tokyo was larger than that of Osaka, the mean DNSMS were consistently lower in the omalizumab group than in the placebo group in Tokyo and Osaka, respectively. Statistically, there was no interaction between the treatment group and the region. Taken to-

Table 2 Drug-related adverse events

		Omalizumab ($n = 48$)	Placebo ($n = 50$)
Total number of patients with ADR*		19 (39.6)	10 (20.0)
Gastrointestinal disorders		1 (2.1)	1 (2.0)
Colitis ulcerative		1 (2.1)	0
Diarrhea		0	1 (2.0)
General disorders and administration site conditions		13 (27.1)	5 (10.0)
Injection site*	Erythema	7 (14.6)	2 (4.0)
	Induration	1 (2.1)	1 (2.0)
	Edema	8 (16.7)	1 (2.0)
	Pain	2 (4.2)	1 (2.0)
	Pruritus	2 (4.2)	0
Feeling hot		1 (2.1)	0
Fatigue		0	1 (2.0)
Pain		1 (2.1)	0
Fever		0	1 (2.0)
Nervous system disorders		0	2 (4.0)
Headache		0	2 (4.0)
Skin and subcutaneous tissue disorders		1 (2.1)	2 (4.0)
Dry skin		1 (2.1)	0
Rash		0	1 (2.0)
Face edema (lip swelling)		0	1 (2.0)
Investigations		4 (8.3) #1	4 (8.0) #2

*: $P < .05$; ↑ : increased; ↓ : decreased

#1: Bilirubin ↑ (1), neutrophil ↓ (1), WBC ↓ (1), WBC ↑ (2)

#2: GPT ↑ (1), eosinophil ↑ (2), lymphocyte ↓ (1), WBC ↑ (1)

gether, regardless of the amount of Japanese cedar pollens, omalizumab would be more effective against SAR.

Our results indicate that subjects treated with omalizumab not only had significantly less severe nasal and ocular symptoms, but also required significantly less rescue medication compared to subjects receiving placebo. In addition, we conducted a double-blind controlled studyusing a competing anti-allergy drug in the next Japanese cedar pollen scattering period, *i.e.*, from February to April 2003. The results showed that omalizumab had significantly lower nasal symptoms and consumption of rescue medications than the competitor (data not shown). Our results suggest that monotherapy with omalizumab at a 2- or 4-week interval can control both nasal and ocular symptoms, thus simplifying SAR therapy.

The omalizumab regimen in the present study was considered appropriate also for Japanese patients with SAR because the regimen successfully decreased serum free IgE levels to below 50 ng/mL, providing proper clinical efficacy, in contrast to the results obtained in foreign studies.

In the omalizumab group, all adverse events except for one (colitis ulcerative) were mild or moderate in severity. The most frequently observed drug-related adverse event in the omalizumab group and the placebo group were injection site reactions, with a significantly higher overall incidence in the former; however, the adverse reaction profile was similar between the two groups when excluding the incidences of injection site reactions. No clinically important abnormal values in laboratory tests or vital signs were reported; no anti-omalizumab antibodies were detected. Furthermore, no cases of anaphylaxis were reported. Therefore, the safety profile of omalizumab in the treatment of SAR seems favorable.

To determine whether omalizumab could consistently provide safety and efficacy in the subsequent season, we conducted an open-label study in the next Japanese cedar pollen scattering period, *i.e.*, from February to April 2003, in order to administer omalizumab to the same subjects who had received the drug in the present study. Consequently, the open-label study revealed no serious adverse events at all and was comparable to the present study with respect to both efficacy and safety (data not shown).

The site of action of omalizumab is localized in free IgE in the circulation, probably local tissues. Omalizumab forms small biologically inert immunocomplexes with free IgE and blocks the interaction between IgE and FcεR which is expressed on the surface of target cells. Additionally, decreases in free IgE levels in microenvironments around mast cells and dendritic cells have been proven to induce the down-regulation of FcεRI expression on the cell surface;[9,21]

the relevant down-regulation is noteworthy because it provides a clinical benefit of possibly reducing the reactivity of mast cells. B lymphocyte apoptosis, the inhibition of IgE production by B lymphocytes,[22,23] and the inhibition of Th2 cytokine production[24] may also be induced by omalizumab treatment. A significant decrease in serum free-IgE levels induced by omalizumab only resembles the transient knockout of IgE because it recovers in a few months after the completion of administration.[12] Considered comprehensively, omalizumab may be potentially beneficial for SAR patients in the clinical settings because it strategically targets sites upstream from the allergic reaction cascade.

Other studies have shown the efficacy of omalizumab for SAR induced by ragweed or birch pollens,[12-14] Recently, its efficacy in perennial allergic rhinitis (PAR)[15] has also been reported. Thus, omalizumab has also clinically been proven to be effective for allergic rhinitis regardless of allergen type and clinical entity. Furthermore, omalizumab induces a non-anaphylactogenic condition, and its combination with specific immunotherapy effectively suppresses enhanced immune responsiveness of patients to a particular allergen and also enhances the efficacy of specific immunotherapy.[25]

In conclusion, omalizumab was well tolerated and effective in preventing and controlling symptoms and in reducing rescue medication use in patients with moderate-to-severe Japanese cedar pollen-induced SAR. Therefore, omalizumab represents a new promising therapeutic modality for patients with SAR induced by Japanese cedar pollens.

REFERENCES

1. Establishment committee of the clinical guideline for allergic rhinitis(2002). [Clinical guideline for allergic rhinitis in Japan.]. *Perennial and seasonal allergic rhinitis*, 4th edn. Tokyo: Life Science Medica, 2002 (in Japanese).
2. Konno A. [Investigation of evaluation of various treatment against pollinosis based on scientific evidence.] *The report of Japanese Ministry of Health and Welfare scientific research for sensory organ impairment and immunoallergy*. 2001 (in Japanese).
3. Okuda M. Epidemiology of Japanese cedar pollinosis throughout Japan. *Ann. Allergy Asthma Immunol.* 2003; **91**:288-296.
4. Kawaguchi T, Hoshiyama Y, Watanabe Y. Cost of cedar pollinosis. *The Allergy in Practice* 2001;**273**:178-182 (in Japanese).
5. Okubo K, Gotoh M, Shimada K *et al.* Fexofenadine improves the quality of life and work productivity in Japanese patients with seasonal allergic rhinitis during the peak cedar pollinosis season. *Int. Arch. Allergy Immunol.* 2005;**136**:148-154.
6. Ishizaka T, Ishizaka K. Activation of mast cells for mediator release through IgE receptors. *Prog. Allergy.* 1984;**34**: 188-235.
7. Presta LG, Lahr SJ, Shields RL *et al.* Humanization of an antibody directed against IgE. *J. Immunol.* 1993;**151**: 2623-2632.
8. MacGlashan DW Jr, Bochner BS, Adelman DC *et al.* Down-regulation of FcεRI expression on basophils during in vivo treatment of atopic patients with anti-IgE antibody. *J. Immunol.* 1997;**158**:1438-1445.
9. Beck LA, Marcotte GV, MacGlashan D Jr, Togias A, Saini S. Omalizumab-induced reductions in mast cell FcεRI expression and function. *J. Allergy Clin. Immunol.* 2004; **114**:527-530.
10. Kolbinger F, Saldanha J, Hardman N, Bendig MM. Humanization of a mouse anti-human IgE antibody: a potential therapeutic for IgE-mediated allergies. *Protein Engineering* 1993;**6**:971-980.
11. Heusser CH, Wagner K, Bews JPA *et al.* Demonstration of the therapeutic potential of non-anaphylactogenic anti-IgE antibodies in murine models of skin reaction, lung function and inflammation. *Int. Arch. Allergy Immunol.* 1997;**113**:231-235.
12. Casale TB, Bernstein L, Busse WW *et al.* Use of an anti-IgE humanized monoclonal antibody in ragweed-induced allergic rhinitis. *J. Allergy Clin. Immunol.* 1997;**100**:110-121.
13. Ädelroth E, Rak S, Haahtela T *et al.* Recombinant humanized mAb-E25, an anti-IgE mAb, in birch pollen-induced seasonal allergic rhinitis. *J. Allergy Clin. Immunol.* 2000; **106**:253-259.
14. Casale TB, Condemi J, LaForce C *et al.* Effect of omalizumab on symptoms of seasonal allergic rhinitis. A randomized controlled trial. *JAMA* 2001;**286**:2956-2967.
15. Chervinsky P, Casale T, Townley R *et al.* Omalizumab, an anti-IgE antibody, in the treatment of adults and adolescents with perennial allergic rhinitis. *Ann. Allergy Asthma Immunol.* 2003;**91**:160-167.
16. Milgrom H, Fick RB, Su JQ *et al.* Treatment of allergic asthma with monoclonal anti-IgE antibody. *N. Engl. J. Med.* 1999;**341**:1966-1973.
17. Soler M, Matz J, Townley R *et al.* The anti-IgE antibody omalizumab reduces exacerbations and steroid requirement in allergic asthma. *Eur. Respir. J.* 2001;**18**:254-261.
18. Busse W, Corren J, Lanier BQ *et al.* Omalizumab, anti-IgE recombinant humanized monoclonal antibody, for the treatment of severe allergic asthma. *J. Allergy Clin. Immunol.* 2001;**108**:184-190.
19. Milgrom H, Berger W, Nayak A *et al.* Treatment of childhood asthma with anti-immunoglobulin E antibody(omalizumab). *Pediatrics* 2001;**108**:1-10.
20. Hochhaus G, Brookman L, Fox H *et al.* Pharmacodynamics of omalizumab: implications for optimised dosing strategies and clinical efficacy in the treatment of allergic asthma. *Curr. Med. Res. Opin.* 2003;**19**:491-498.
21. Prussin C, Griffith DT, Boesel KM, Lin H, Foster B, Casale TB. Omalizumab treatment downregulates dendritic cell FcRεI expression. *J. Allergy Clin. Immunol.* 2003;**112**:1147-1154.
22. Chang TW. The pharmacological basis of anti-IgE therapy. *Nature Biotechnology* 2000;**18**:157-162.
23. Shields RL, Whether WR, Zioncheck K *et al.* Inhibition of allergic reactions with antibody to IgE. *Int. Arch. Allergy Immunol.* 1995;**107**:308-312.
24. Coyle AJ, Wagner K, Bertrand C, Tsuyuki S, Bews J, Heusser C. Central role of immunoglobulin (Ig) E in the reduction of lung eosinophil infiltration and T helper 2 cell cytokine production : inhibition by a non-anaphylactogenic anti-IgE antibody. *J. Exp. Med.* 1996; **183**:1303-1310.
25. Kuehr J, Brauburger J, Zielen S *et al.* Efficacy of combination treatment with anti-IgE plus specific immunotherapy in polysensitized children and adolescents with seasonal allergic rhinitis. *J. Allergy Clin. Immunol.* 2002;**109**:274-280.

Allergy 2007: 62: 547–553

DOI: 10.1111/j.1398-9995.2007.01331.x

Original article

Isolation and characterization of native Cry j 3 from Japanese cedar (*Cryptomeria japonica*) pollen

Background: Japanese cedar (*Cryptomeria japonica*) pollinosis is the most prevalent allergy in Japan. Recently, the Japanese cedar pollen allergen Cry j 3 was cloned as a homologue of Jun a 3, which is a major allergen from mountain cedar (*Juniperus ashei*) pollen. However, native Cry j 3 has not been isolated and there are no reports on its allergenic activity. The aims of this study were to isolate native Cry j 3 and assess its immunoglobulin E (IgE)-binding capacity in patients with Japanese cedar pollinosis.
Methods: Native Cry j 3 was purified from Japanese cedar pollen by multidimensional chromatography. We assessed the IgE-binding capacity using sera from patients allergic to Japanese cedar pollen by immunoblot analysis and ELISA. Moreover, we assayed the capacity of Cry j 3 to induce histamine release from the patients' leukocytes. We cloned cDNA corresponding to purified Cry j 3 from a cDNA library of Japanese cedar pollen.
Results: We isolated native Cry j 3 as a 27-kDa protein. The IgE-binding frequency of Cry j 3 from the sera of patients allergic to Japanese cedar pollen was estimated as 27% (27/100) by ELISA. Cry j 3 induced the release of histamine from leukocytes. We cloned the cDNA and named it Cry j 3.8. Cry j 3.8 cDNA encoded 225 amino acids and had significant homology with thaumatin-like proteins.
Conclusions: Cry j 3 is a causative allergen in Japanese cedar pollinosis and may play crucial roles in the cross-reactivity with oral allergy syndrome.

T. Fujimura[1], N. Futamura[2], T. Midoro-Horiuti[3], A. Togawa[3], R. M. Goldblum[3], H. Yasueda[4], A. Saito[4], K. Shinohara[2], K. Masuda[5], K. Kurata[5], M. Sakaguchi[1]

[1]Graduate School of Medicine, Chiba University, Chiba; [2]Department of Molecular and Cell Biology, Forestry and Forest Products Research Institute, Ibaraki, Japan; [3]Department of Pediatrics, Child Health Research Center, University of Texas Medical Branch, Galveston, TX, USA; [4]Clinical Research Center for Allergy and Rheumatology, Sagamihara National Hospital, Kanagawa; [5]Research Center for Allergy and Immunology, Yokohama Institute, RIKEN (The Institute of Physical and Chemical Research), Kanagawa, Japan

Key words: Japanese cedar; oral allergy syndrome; pathogenesis-related protein; pollinosis; thaumatin-like protein.

Dr Masahito Sakaguchi
Graduate School of Medicine
Chiba University
1-8-1, Inohara
Chuo-ku
Chiba
Japan

Accepted for publication 27 December 2006

Japanese cedar (*Cryptomeria japonica*) pollen is one of the most prevalent allergens in Japan. A nationwide survey using a cross-sectional random sampling method estimated that at least 13% of the Japanese population is suffering from Japanese cedar pollinosis (1). Because of the steady increase in this allergy, especially among schoolchildren, Japanese cedar pollinosis has become a severe social problem.

Two major allergens from Japanese cedar pollen, Cry j 1 and Cry j 2, have been shown to cause Japanese cedar pollinosis (2, 3). Cry j 1 was identified as a 41–46 kDa allergen with pectate lyase enzyme activity (2, 4, 5), while Cry j 2 was identified as a 45 kDa allergen with polymethylgalacturonase enzyme activity (3, 6–8). Both Cry j 1 and Cry j 2 have several isoforms that differ in their primary structures, post-translational modifications, or reactivity with antibodies (9–11).

Recently, we cloned seven isoforms of cDNA encoding Cry j 3 as homologues of Jun a 3 (12, 13). Jun a 3 is a major allergen from mountain cedar (*Juniperus ashei*) pollen and has been reported as a pathogenesis-related-5 group family (PR-5) protein allergen with an immunoglobulin E (IgE)-binding reactivity of 42.9% (6/14) in patients allergic to mountain cedar pollen. Moreover, Jun a 3 has been reported to cross-react in pollinosis patients allergic to Japanese cedar pollen at a rate of 33.3% (12/36) (14). This suggests that Cry j 3 has IgE-binding capacity in Japanese cedar pollinosis patients and cross-reacts with Jun a 3. However, the native protein of Cry j 3 has not been isolated, and thus its IgE-binding capacity has not been elucidated despite its importance as a PR-5 family allergen. Therefore, we isolated native Cry j 3 from Japanese cedar pollen and assessed its IgE-binding capacity.

In the present study, we determined the IgE-binding capacity of native Cry j 3 in patients allergic to Japanese cedar pollen using immunoblot, ELISA and histamine-release assays. We also cloned the cDNA corresponding to the isolated novel isoform of Cry j 3, and named it Cry j 3.8.

Materials and methods

Purification of Cry j 3

Crude extracts were obtained from Japanese cedar pollen as described previously (2). Briefly, Japanese cedar pollen was suspended in 125 mM sodium bicarbonate buffer (pH 8.0) containing 3 mM ethylenediamine tetraacetic acid for 4 h at 4°C, and the suspension was centrifuged at 9300 *g* for 35 min at 4°C. Ammonium sulfate was added to the supernatant until 80% saturation. The resultant precipitate was dialyzed against 5 mM phosphate buffer (pH 6.8) and then centrifuged at 10 000 *g* for 15 min at 4°C. The supernatant was filtered through a 1.0 μm pore membrane filter (Toyo Roshi Ltd, Tokyo, Japan) to obtain crude Japanese cedar pollen extract. The crude extract was applied directly to a DEAE-Cellulofine column (Seikagaku Corporation, Tokyo, Japan). The unadsorbed fraction was applied to a Micro-Prep® Ceramic Hydroxyapatite type I column (BioRad Laboratories Inc., Hercules, CA, USA), and then ammonium sulfate was added to the unadsorbed fraction until 80% saturation. The resultant precipitate was dialyzed against 20 mM acetate buffer (pH 5.0). The dialyzed solution was applied to a Hiload 26/10 SP Sepharose HP column (GE Healthcare Bio-Sciences Corporation, Piscataway, NJ, USA). The obtained unadsorbed fraction was dialyzed against 0.065% trifluoroacetic acid in 2% acetonitrile, and then applied to a Resource™ RPC column (GE Healthcare Bio-Sciences Corporation). The adsorbed fraction was obtained by gradient elution with 0.05% trifluoroacetic acid in 80% acetonitrile. The fractions containing native Cry j 3 were pooled and phosphate-buffered saline (PBS) was used as an exchange buffer in a PD-10 desalting column (GE Healthcare Bio-Sciences Corporation). The resultant single protein was named Cry j 3. N-terminal amino acid sequencing of purified Cry j 3 was performed by Toray Research Center Inc., Tokyo, Japan.

SDS-PAGE and immunoblotting

Protein samples (crude pollen extract and purified Cry j 3) were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12.5% slab gel under reducing conditions with 50 mM dithiothreitol using the discontinuous buffer system of Laemmli (15). Proteins were then detected with PhastGel™ Blue R (GE Healthcare Bio-Sciences Corporation) or blotted onto a Hybond-P membrane (GE Healthcare Bio-Sciences Corporation) at 1 mA/cm^2 for 1.5 h. The blot was probed with primary antibody (anti-Jun a 3 rabbit serum IgG, or sera from Japanese cedar pollinosis patients or healthy individuals). To detect rabbit IgG, the blot was incubated with horseradish peroxidase-conjugated anti-rabbit IgG (Zymed Laboratories Inc., San Francisco, CA, USA). In the case of human IgE, the blot was overlaid with biotinylated anti-human IgE (Vector Laboratories Inc., Burlingame, CA, USA), followed by reaction with horseradish peroxidase-conjugated streptavidin (Zymed Laboratories). After immunolabeling, the positive bands were visualized on the membrane using 3,3′,5,5′-tetramethylbenzidine or on a chemiluminescence imager (Atto Corp., Tokyo, Japan) or an X-ray film (Fuji Photo Film Co. Ltd, Tokyo, Japan) using an ECL-Plus Western blotting detection kit (GE Healthcare Bio-Sciences Corporation).

Analysis of glycosylation on Cry j 3

Five micrograms of protein samples (Cry j 1, Cry j 3, horseradish peroxidase as a positive control and soybean trypsin inhibitor as a negative control) were fractionated by SDS-PAGE, followed by blotting onto polyvinylidene difluoride (PVDF) membrane as described above. Glycoprotein was visualized by using a GelCode™ Glycoprotein staining kit (PIERCE Biotechnology, Inc., Rockford, IL, USA) according to the manufacturer's instruction. Briefly, gel was incubated with oxidizing solution and then washed three times by gently agitating with 3% acetic acid. The gel was submerged in glycoprotein staining reagent, followed by reaction with reducing solution with gentle agitation. The gel was washed with 3% acetic acid, followed by ultrapure water.

ELISA for specific IgE to pollen allergens

Specific IgE to pollen allergens was measured by fluorometric ELISA. Briefly, purified antigen solutions (500 ng/ml of Cry j 1, Cry j 2 or Cry j 3) were applied to a 96-well microtiter plate (NUNC-Immuno® Plate Maxisorp F96; NalgeNunc International, Roskilde, Denmark) and incubated at 4°C overnight. After the plate was blocked with 1% (w/v) bovine serum albumin in PBS for 2 h at 37°C, 10-fold diluted patients' sera were added and incubated for 4 h at room temperature. Diluted (1 : 10) β-galactosidase-conjugated anti-human IgE monoclonal antibody (Pharmacia Diagnostics AB, Uppsala, Sweden) was then added, followed by incubation at 4°C overnight. For enzymatic reaction, 0.2 mM 4-methylumbelliferyl β-D-galactopyranoside (Sigma Aldrich Corp., St Louis, MO, USA) was added, followed by incubation at 37°C for 2 h. The fluorescence intensity was measured using a fluorometric microplate reader (Fluoroscan; Flow Laboratories, McLean, VA, USA).

Assay of histamine release from human leukocytes

Histamine release experiments from washed leukocytes were conducted using the same method as described previously (16). Washed leukocytes were obtained from the venous blood of donors and suspended in PIPES buffer (25 mM piperazine-*N*,*N*′-bis-2-ethanesulfonic acid, 110 mM sodium chloride and 5 nM potassium chloride) containing 1 mM Ca^{2+}, 1 mM Mg^{2+} and 0.03% (w/v) human serum albumin (pH 7.4). Allergen preparations diluted in the same PIPES buffer were mixed at a series of concentrations with washed leukocytes at 37°C for 45 min. The histamine released into the supernatant was measured by automated fluorometry (17).

Molecular cloning of Cry j 3.8 from a cDNA library

We constructed a full-length cDNA library from male flowers and pollen of Japanese cedar at several stages during the development (N. Futamura et al., unpublished data). In addition, the 5′ ends of cDNA sequences from approximately 20 000 clones obtained from the cDNA library were analyzed. We obtained a clone in which the 5′-end sequence coincided completely with the sequence deduced from the N-terminal amino acid sequence of purified native Cry j 3. Thus, we determined the complete cDNA sequence from the clone and named it Cry j 3.8.

Results

Purification of Cry j 3 from Japanese cedar pollen

As in the many cases of purification of thaumatin-like proteins (TLPs), we isolated a single protein after gradient elution with acetonitrile on reverse phase chromatography following anion exchange, hydroxyapatite and cation exchange chromatography. The purified protein migrated at 27.3 kDa under reducing conditions on SDS-PAGE (Fig. 1A). The homologous purified protein reacted with anti-Jun a 3 polyclonal antibody on Western blotting (Fig. 1B). To confirm that this positive protein was Cry j 3, we analyzed the N-terminal amino acid sequence, which was found to coincide completely with that of Jun a 3. Therefore, we concluded that the purified protein was native Cry j 3 (Fig. 2).

Determination of Cry j 3-specific IgE levels in the sera of patients allergic to Japanese cedar pollen

Purified Cry j 3 on a blot was reactive with the sera from patients allergic to Japanese cedar pollen but not with the sera from nonallergic individuals (Fig. 3A). ELISA was performed using the sera of 100 Japanese cedar pollinosis patients to analyze the IgE-binding frequency and specific IgE levels against Cry j 3. Twenty-seven percent (27/100) of these patients exhibited significantly greater IgE-binding ability against Cry j 3 compared with healthy volunteers (Fig. 3B). The IgE-binding intensity of Cry j 3 in the patients' sera was almost one 14th and one 10th of the IgE-binding intensities of Cry j 1 and Cry j 2, respectively (the mean fluorescence intensities with patients' IgE against Cry j 1, Cry j 2 and Cry j 3 were 5800, 3900 and 410, respectively; Fig. 3C).

Purified Cry j 3 induced histamine release from leukocytes obtained from Cry j 3-positive patients in a dose-dependent manner, but not from Cry j 3-negative patients (Fig. 4). The sensitivity (the dose of allergen that induced 25% histamine release) was one 15th that of Cry j 1 (32 ng/ml for Cry j 1 and 480 ng/ml for Cry j 3; Fig. 4A). These data were consistent with the difference in IgE-binding intensity between Cry j 3 and Cry j 1 (1 : 14).

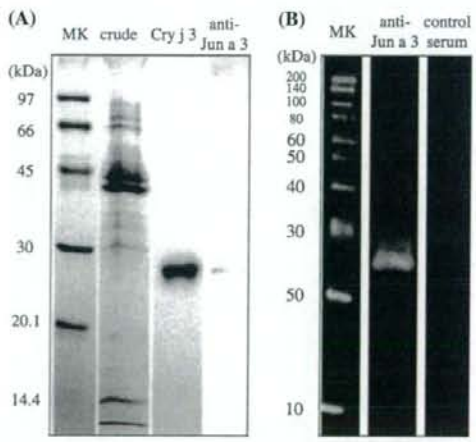

Figure 1. Purification of native Cry j 3 from crude Japanese cedar pollen extract. Crude extract from Japanese cedar pollen and purified Cry j 3 were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The molecular weight marker (lane 1), crude soluble extract (lane 2) and purified Cry j 3 (lane 3) were separated. Crude soluble extract was then transferred onto polyvinylidene difluoride (PVDF) membrane, followed by staining with anti-Jun a 3 rabbit serum (lane 4) (A). Cry j 3.8 was subjected to SDS-PAGE and transferred onto a PVDF membrane. The blots were stained with anti-Jun a 3 rabbit serum or control rabbit serum (B).

```
Cry j 3   : V K F D I K N Q C G Y T V
Jun a 3   : V K F D I K N Q C G Y T V
Cry j 3.1: A T F D I T N Q C P Y T V
Cry j 3.2: A T F D I T N Q C P Y T V
Cry j 3.3: A T F D I T N Q C P Y T V
Cry j 3.4: V N F D I E N Q C P Y T V
Cry j 3.5: T V F T L V N K C S Y T V
Cry j 3.6: P T F E I T N K C P Y T V
Cry j 3.7: V K F E L K N Q C E Y T V
```

Figure 2. Alignment of N-terminal amino acid sequences from Cry j 3 isoforms and Jun a 3.

Molecular cloning of Cry j 3.8 from Japanese cedar pollen

The N-terminal sequence of the purified protein did not coincide with the deduced amino acid sequences from any of the previously cloned Cry j 3 isoforms, Cry j 3.1 to Cry j 3.6 (12, 13) and Cry j 3.7 (accession number AB212218) (Fig. 2). Therefore, we again cloned the cDNA from the cDNA library of Japanese cedar and named the novel isoform Cry j 3.8 (accession number AB254807).

Sequence analysis revealed that the Cry j 3.8 cDNA consisted of 943 nucleotides containing a 678 bp open reading frame (ORF). The ORF encoded a 225 amino acid polypeptide. In addition, we found two potential N-glycosylation sites, one consensus pattern of the TLP family, and 16 consensus cysteine residues in the ORF (18, 19) (Fig. 5). By glycoprotein staining of Cry j 3, we detected a faint positive band on the blot, indicating that native Cry j 3 was actually glycosylated (data not shown). The deduced amino acid sequence of whole Cry j 3.8 had 45–76% identity with other Cry j 3 isoforms and 86% identity with Jun a 3. Multiple sequence alignment showed a high degree of identity between Cry j 3.8 and other TLPs, especially with previously described TLP allergens from other plant species (Fig. 6). The amino acid identities between Cry j 3.8 and the other TLP allergens, Jun r 3.1 (*J. rigida*), Jun r 3.2, Jun a 3, Cup s 3.2 (*Cupressus sempervirens*) and Cup s 3.3 were 86.2%, 85.8%, 85.8%, 84.9% and 84.9%, respectively.

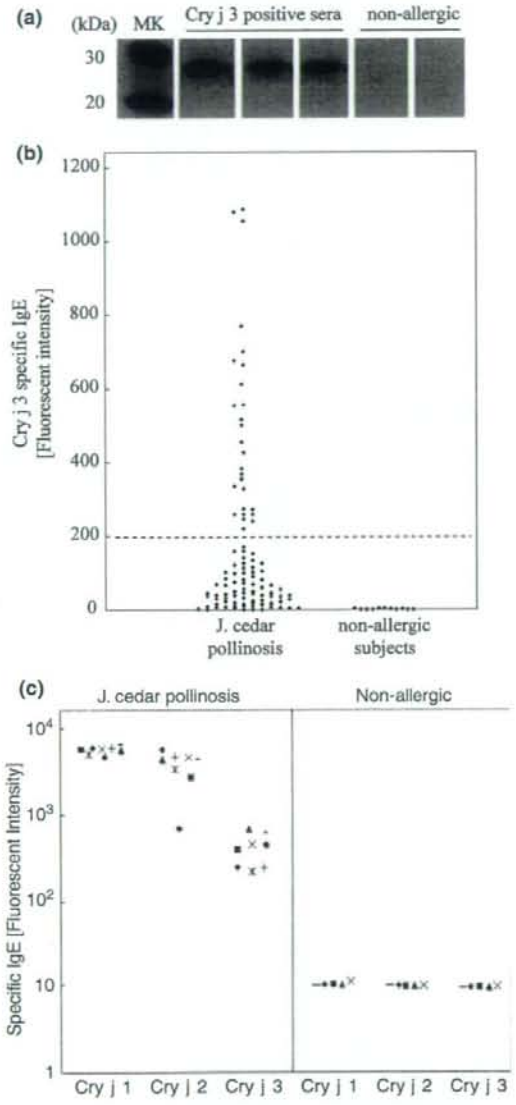

Figure 3. Immunoglobulin E (IgE)-binding ability of Cry j 3 in patients allergic to Japanese cedar pollen. Purified Cry j 3 was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane. The blots were stained with serum IgE from Japanese cedar pollen-sensitized patients or healthy subjects as controls (A). ELISA was performed using IgE in the sera from 100 patients with Japanese cedar pollinosis and 11 healthy subjects. The cut line shows the cut-off value for a positive result (B). IgE-binding capacity was compared among Cry j 1, Cry j 2 and Cry j 3 (C). The specific IgE level against each allergen was assayed by ELISA using sera from eight Cry j 3.8-positive allergic patients with Japanese cedar pollinosis and five nonallergic subjects as controls. Identical symbols show IgE titers from the same patient or healthy subject.

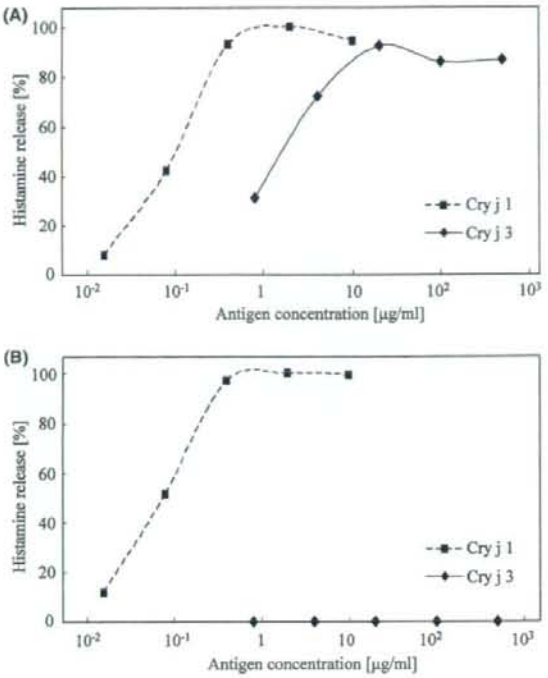

Figure 4. The amount of histamine released into the culture supernatant from leukocytes obtained from Cry j 3-positive (A) and Cry j 3-negative (B) Japanese cedar pollinosis patients stimulated with Cry j 1 or Cry j 3 was assayed by automated fluorometry.

Discussion

The IgE-binding frequency of Cry j 3 in pollinosis patients allergic to Japanese cedar pollen was estimated as 27%. The IgE-binding capacity was estimated as one 15th when compared with Cry j 1 by ELISA and histamine-release assay from leukocytes, and one 10th when compared with Cry j 2 by ELISA. In one patient, the specific IgE titer against Cry j 3 was comparable to that against Cry j 2 (filled circles in Fig. 3C). This suggests that Cry j 3 is an important allergen in some patients. It has been reported that the expression level and allergenic activity of TLPs vary under different environmental conditions (20). Therefore, the importance of Cry j 3 as a Japanese cedar allergen may differ among years and locations.

Cry j 1 cross-reacts with Jun a 1, and the two allergens share some linear and probable conformational epitopes (21). Moreover, cross-reactivity between Cry j 3 and Jun a 3 has also been predicted. Indeed, in the present study the anti-serum raised against Jun a 3 recognized Cry j 3, and it has been reported that 33% (12/36) of a group of patients allergic to Japanese cedar pollen showed