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The role of costimulatory molecules (B7-1 and B7-2) on allergen-stimulated B cells in cedar pollinosis subjects

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Summary

Background B7-1 (CD80) and B7-2 (CD86), which are costimulatory molecules in T-cell activation, play important roles in the differentiation of TH1- or TH2-phenotypes. These molecules were also suggested to play important roles in sensitization to a cedar pollen antigen by blocking studies using neutralizing antibodies, but there have been very few studies concerning the effects following induction by antigen.

Objective In this study, we investigated the roles of B7-1 and B7-2 in the differentiation of TH1 and TH2 subsets after stimulation with the antigen in subjects with cedar pollinosis.

Methods Skin-prick test was performed in nine subjects with pollinosis and in nine normal controls. Peripheral blood mononuclear cells (PBMCs) were isolated and stimulated with Japanese cedar pollen extract. After *in vitro* stimulation, the expression of CD80 and CD86 on $CD19^+$ cells was analysed by two-colour flow cytometry. Culture supernatants were collected for all subjects and the production of type 1 and type 2 cytokines was measured by ELISA.

Results After *in vitro* stimulation, the expression of CD80 (B7-1) was upregulated in both pollinosis and control subjects, but no significant difference was observed between the two groups. On the other hand, CD86 (B7-2) was significantly upregulated following stimulation in pollinosis subjects ($P = 0.02$). A significantly higher level of IL-5 ($P = 0.04$) was produced by PBMCs of pollinosis subjects than by those of normal controls. A significantly higher level of interferon (IFN)-γ ($P = 0.03$) was produced by PBMCs of normal controls than by those of pollinosis subjects.

Conclusion These results indicated that TH2 response was predominant in pollinosis subjects, and that $CD19^+$ cells of pollinosis subjects expressed higher levels of B7-2 than those of control subjects after *in vitro* stimulation. In pollinosis subjects, B7-2 rather than B7-1 may be the costimulatory molecule involved in allergen-induced activation of PBMCs.

Keywords: cedar pollinosis, costimulatory molecules, cytokine production, TH2 response

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Introduction

T-lymphocyte activation *in vitro* requires at least two signals. The first signal is generated by the T-cell receptor (TCR)/CD3 complex after engagement with a nominal Ag presented in contact of major histocompatibility (MHC) proteins. However, delivery of the first signal is not sufficient to activate T cells *in vitro* [1–4]. For T-cell activation, the second signal is necessary. Both cell-bound accessory molecules and soluble cytokines are known to act as costimulatory molecules [3,4]. In the absence of the second signal, TCR signalling can induce a long-lasting anergic state in which appropriate presentation of Ag fails to elicit interleukin (IL)-2 production or T-cell proliferation [4]. Recent studies have indicated that CD28 and CTLA-4 function as T-cell accessory molecules capable of delivering the appropriate costimulatory signals to permit T-cell

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activation and prevent the induction of tolerance [5–7]. Stimulation of CD28 working with the signal through TCRs induces the secretion of several cytokines such as IL-2 [8–10]. CD28 and CTLA-4 bind to at least two ligands on antigen-presenting cells (APCs), the B7-1 and B7-2 molecules [1,2,11]. B7 is now recognized as a family of receptors expressed on specialized APCs such as activated T and B cells, macrophages, foetal and neonatal thymic epithelial cells and dendritic cells, which have been shown to play a critical role in T-cell activation [6]. The interaction of CD28/CTLA-4 on T cells with B7 on APCs constitutes an important costimulatory signal for T cells in the prevention of anergy induction after T-cell receptor triggering [4–7]. Recently, there has been a great deal of interest in whether B7-1 and B7-2 play distinct roles in differentiation of TH subsets [12–14]. Following antigenic stimulation, $CD4^+$ TH cells differentiate into subpopulations producing distinct spectra of cytokines with different functions [15]. TH1 cells are characterized by IL-2, TNFβ and IFNγ production, and aid in cell-mediated immunity. In contrast, TH2 cells are characterized by IL-4, IL-5 and IL-10 production, and aid in humoral immunity (especially the production of IgE and IgG1 isotypes) and possibly suppression of TH1-type responses [16,17]. Recent studies have suggested that B7-1 and B7-2 play distinct roles in the induction of TH1 and TH2 responses. The differential expression of B7-1 and B7-2 in addition to the observation that these molecules bind to distinct determinants on CD28 suggested that they may also have functional differences. In fact, murine B7-1 and B7-2 only share 25% amino acid homology and have marked differences in their cytoplasmic domains [18]. In addition, murine B7-2 is expressed constitutively on professional APCs and is rapidly induced in response to a variety of stimuli [19,20]. B7-1, on the other hand, is expressed much later after activation and also at lower levels than B7-2 [20]. However, studies of the roles of CD28/B7 molecules in differentiation of TH cells have yielded contradictory results. Kuchroo *et al*. reported that blockade of B7-1 ameliorated experimental autoimmune encephalomyelitis (EAE), a TH1-mediated disease, whereas blockade of B7-2 exacerbated this disease [21]. In contrast, anti-B7-2 treatment was seen to depress the onset of diabetes in nonobese diabetic (NOD) mice, whereas anti-B7-1 treatment exacerbated the disease [22]. These findings suggested that the relationship between B7-1/B7-2 and cytokine development is not as straightforward as previously thought. There is now extensive evidence implicating TH2 cells as key mediators of the chronic inflammatory process that underlies many allergic diseases including asthma [23]. Japanese cedar pollinosis is an atopic disease similar to asthma. Although it is still not clear whether B7-1 and B7-2 costimulation cause differentiation of T cells into TH1 and TH2, several investigators have reported a role of B7-2 in differentiation of naive T cells into the TH2 subsets [24–26]. In this study, we examined the roles of the costimulatory molecules B7-1 and B7-2 on $CD19^+$ cells in subjects with pollinosis.

Materials and methods

Blood donors

Blood samples were collected from nine patients with Japanese cedar pollinosis and nine normal healthy control subjects during the Japanese cedar pollen season (Table 1). All patients had a clinical history of allergy to Japanese cedar pollen. No patients had received specific immunotherapy. Skin-prick testing was performed (wheal diameter at least 4 mm greater than negative controls) with commercial crude extracts used *in vivo* and *in vitro* (Torii Pharmaceuticals, Tokyo, Japan). No patients showed skin reactions when tested with SoluPrick dilution as a negative control. Normal healthy donors had no history of allergic symptoms and exhibited negative skin prick tests in this study. All subjects gave their informed consent to participate in this study, and the protocol was approved by the Dokkyo Medical School Committee on the use of Human Subjects in Research (Tochigi, Japan).

Isolation of PBMCs

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood samples by Ficoll-density gradient centrifugation, and the interface was collected. Following two washes with the serum-free medium, the cells were further incubated in RPMI 1640 medium (Gibco Life Technologies, Grand Island, NY, USA), supplemented with 10% (v/v) foetal calf serum (Gibco), 50 U/mL penicillin-G, 50 μg/mL streptomycin and 2 mmol/L L-glutamine (Sigma, St Louis, MO, USA) (complete medium) at a concentration of 1.5×10^6 cells/mL in 12-well plates (Iwaki Glass Co., Chiba, Japan) for 3 h at 37 °C. As most blood samples were collected in the pollinosis season, PBMCs might have already been exposed to cedar pollen antigen at the start of our study. Therefore, all PBMCs were analysed by flow cytometry before and after stimulation. Nonadherent cells containing the T-cell population were harvested and analysed by flow cytometry as PBMCs before stimulation. For further studies, PBMCs were taken following density gradient and used directly in experiments as described below.

Primary bulk culture

Cells were suspended in complete medium and cultured at a concentration of 1.5×10^6 cells/mL in flat-bottomed 12-well plates at 37 °C in a fully humidified atmosphere 5% CO_2 in

Table 1. Profiles of the blood donors*

	Sex	Age	Skin-prick test†	IgE RAST	Complication	Treatment
Pollinosis subject						
1	M	26	+++	Cedar, mite, HD	None	Anti-allergic drug
2	F	32	+++	Cedar	Atopic dermatitis	Anti-allergic drug
3	F	38	++	Cedar, cat, HD	Atopic dermatitis	Anti-allergic drug
4	F	25	+	Cedar, HD	None	Anti-allergic drug
5	M	38	++	Cedar, mite, HD	None	Anti-allergic drug
6	M	25	++	Cedar, mite, HD	Atopic dermatitis	Anti-allergic drug
7	F	37	+	Cedar, mite, HD	None	Anti-allergic drug
8	M	30	++	Cedar	None	Anti-allergic drug
9	M	27	+++	Cedar, cat, HD	None	Anti-allergic drug
Control subjects						
1	M	38	–	–	None	None
2	M	25	–	–	None	None
3	M	25	–	–	None	None
4	M	26	–	–	None	None
5	M	24	–	–	None	None
6	M	31	–	–	None	None
7	M	47	–	–	None	None
8	M	26	–	–	None	None
9	M	47	–	–	None	None

*Blood samples from nine allergic patients with Japanese cedar pollinosis and nine normal control subjects were collected as described in 'Materials and methods'. †+: weal reaction 9–12 mm or flare reaction 20–29 mm, ++: weal reaction 9–12 mm and flare reaction 20–29 mm, +++: weal reaction > 13 mm and flare reaction > 30 mm. HD, house dust.

air for 6 days. Following initial optimization of the antigen concentration and culture duration, short-term cultures were set up for each subject in the presence of Japanese cedar pollen extract at a dilution of 1 : 50, which contained 120 ng/mL of Cry j 1. The same allergen extract was used for skin testing and for *in vitro* culture. After 6 days, the cells were harvested by centrifugation and immediately stained with antibodies for FACS analysis. Culture supernatants were collected, filtered and stored at − 70 °C until analysis of IL-4, 5 and IFNγ production.

FACS analysis of PBMCs

Two-colour flow cytometry analysis was performed as previously described [27] using a modified Dual-Laser FACScan Plus (Becton Dickinson, Immunocytometry Systems, San Jose, CA, USA). Monoclonal antibodies conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE) were used. FITC-conjugated anti-CD4, and PE-conjugated anti-CD19 and anti-CD25 for IL-2Rα were purchased from DAKO (Glostrup, Denmark). PE-conjugated anti-CD152 for CD40L and FITC-conjugated anti-CD40, CD80 for B7-1 and CD86 for B7-2 were purchased from Pharmingen (San Diego, CA, USA). PE-conjugated anti-CD28 and anti-CD154 for CTLA-4 were purchased from Becton Dickinson. In all experiments, isotype-matched antibodies purchased from Pharmingen were used as controls. Gates for positive and negative populations were set in a similar fashion for all groups. For staining, cell suspensions were adjusted to the concentration of 1×10^6 cells/mL in 0.1% (w/v) sodium azide-PBS, and incubated with pre-titred mAbs for 30 min at 4 °C. Cells were washed twice with 0.1% sodium azide-PBS, and fixed with 1% (w/v) paraformaldehyde-PBS. Analysis was performed within 24 h.

Cytokine quantification in culture supernatants

Levels of IL-4, IL-5 and IFNγ in the culture supernatants were measured in duplicate by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol (Amersham, Amersham, UK).

Statistical analysis

The non-parametric Mann–Whitney *U*-test was used to compare the expression of surface markers and cytokine levels in patients with controls. The comparison of mean

fluorescence intensity (MFI) after stimulation and MFI before stimulation was performed using the Student's *t*-test. Analysis was performed using a statistical software package (Statview, Abacus Concepts Inc., Berkeley, CA, USA). Differences were considered significant when *P*-values were < 0.05.

Results

Visual observation of PBMCs cultured for 6 days indicated the presence of two cell types, i.e. small and large cells. The large cells within the gated area were used for analysis, because lymphocytes activated by antigen are increased in size. These observations were confirmed by flow cytometry, which demonstrated the presence of two clear populations defined by different forward and side scattering profiles (Fig. 1). On average, the large cells (within the gate area) constituted 8–10% of the total viable cell population (data not shown). Immunolabelling was carried out on these unseparated mixed populations, and then only large cell populations, which were continuous from the main populations, were analysed using the gates as shown in Fig. 1. In this gated area, mean percentages of $CD4^+$ cells and $CD19^+$ cells were 45 and 31% in allergen-stimulated PBMCs from pollinosis subjects, and 54 and 34% in those from control subjects, respectively. For each surface marker as described below, upregulation ratio (MFI after stimulation/MFI before stimulation) was compared between pollinosis and control subjects.

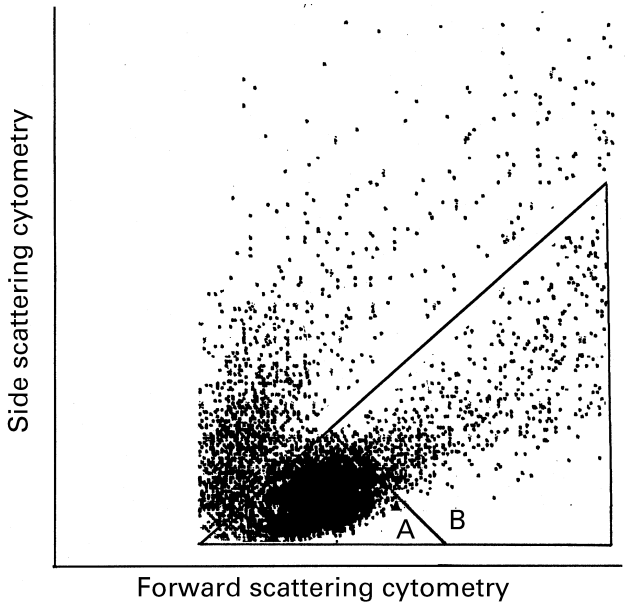


Fig. 1. Flow cytometry analysis of a pollinosis subject. The presence of two clear populations was defined by different forward and side scattering profiles. Immunolabelling was carried out on these unseparated mixed populations (area A + B), and then only large cell populations (area B) were analysed using the gates

$CD4^+$ cells in both groups expressed low levels of CD28 and CD152 (CTLA-4) before stimulation. After *in vitro* stimulation, the expression of CD28 and CTLA-4 was upregulated in both pollinosis and control subjects, with no significant differences between the two groups (Fig. 2).

Before stimulation, $CD19^+$ cells in both groups expressed low levels of CD80 (B7-1) and CD86 (B7-2). After *in vitro* stimulation, the expression of B7-1 was upregulated in both pollinosis and control subjects, and no significant difference was observed between the two groups (Fig. 3a). CD86 (B7-2) was also upregulated upon stimulation in pollinosis subjects after *in vitro* stimulation (Fig. 3b). The $CD19^+$ cells of pollinosis subjects expressed CD86 more strongly than controls ($P = 0.02$).

The expression of CD86 on $CD19^+$ cells before and after *in vitro* stimulation was also compared between the two groups. No significant difference in CD86 expression between the two groups was observed before stimulation, whereas the difference between them was statistically significant after stimulation ($P = 0.03$).

Both groups expressed low levels of CD154 (CD40L) before stimulation. After *in vitro* stimulation, the expression of CD40L was upregulated in both pollinosis and control subjects, and no significant difference was observed between the two groups (Fig. 4b). After *in vitro* stimulation, $CD19^+$ cells from pollinosis subjects expressed CD40 more strongly than those of control subjects (Fig. 4a), but the difference between the two groups was not statistically significant.

To investigate the specificity of immune responses to allergen, the same analysis was performed using PBMCs cultured for 6 days without allergen. After cultivation with medium alone, no statistically significant differences in the expression of any molecules were observed between pollinosis and control subjects (data not shown).

To characterize the T cells responding to Japanese cedar pollen extract, PBMCs were stimulated with the allergen *in vitro* for 6 days, and the amounts of IL-4, IL-5 and IFNγ in the culture supernatants were measured by ELISA. The sensitivities of the assays were 40 pg/mL for IL-4, and 2 pg/mL for IL-5 and IFNγ. IL-4 production was detected in neither allergic nor normal control groups. As shown in Fig. 5(a), significant numbers of pollinosis subjects (eight of nine) produced IL-5 in the presence of the cedar pollen antigen, whereas only two of eight normal controls produced detectable amounts of this cytokine. The mean concentration of IL-5 was 136.1 pg/mL for the pollinosis subjects and 8.3 pg/mL for the normal controls. This difference in level of IL-5 production by PBMCs between the two groups was significant ($P = 0.04$). Eight of nine allergic subjects produced IFNγ with a mean concentration of

100.3 pg/mL, whereas all of eight normal control subjects contained detectable levels of IFNγ with a mean concentration of 295.8 pg/mL. This difference between two the groups was also significant ($P = 0.03$).

Discussion

A number of recent studies have demonstrated the costimulator-dependence of allergen-specific human T cells and the role of these CD28/CTLA-4 and CD80 (B7-1)/CD86 (B7-2) in animal models of allergic diseases. The role of B7-1/B7-2 in the differentiation of T helper ($CD4^+$) lymphocytes, directing them towards either a TH1- or TH2-type phenotype has been a controversial issue. We examined the role of B7-1 and B7-2 costimulatory molecules in the differentiation of TH1 and TH2 subsets in pollinosis subjects. T-cell proliferation does not occur until an additional, costimulatory signal is received. This second signal is provided by surface ligands on antigen-presenting cells (APCs). The identification of B7 molecules on the surface of APCs and their interaction with CD28 on the surface of T cells led to the discovery that the B7-CD28 pathway plays a role in costimulation of the T-cell response [28]. After T-cell activation, the expression of CD28 is increased. Once a signal is initiated through the T-cell receptor, ligation of CD28 induces upregulation of IL-2 production and increases T-cell proliferation [29,30].

CD28 costimulation may be more important for the development of TH2 responses which are dependent on higher doses of antigen, while TH1 responses seem to have less of a requirement for CD28 costimulation [31]. CD28-deficient mice have been reported to have enhanced levels of TH1-type cytokines and diminished TH2-type cytokine levels in a model of autoimmune diabetes [22]. This result suggested the dependence of CD28 signalling for differentiation of the TH2 subset of naive diabetogenic T cells to occur early in the course of the disease. CD28-deficient mice have decreased TH2 responses to *Schistosoma mansoni* eggs along with diminished IL-4 and IL-5 levels [32]. It is now accepted that a major T-cell costimulatory pathway involves the CD28 molecule. CD28 interactions with the B7 family of costimulatory ligands are essential for

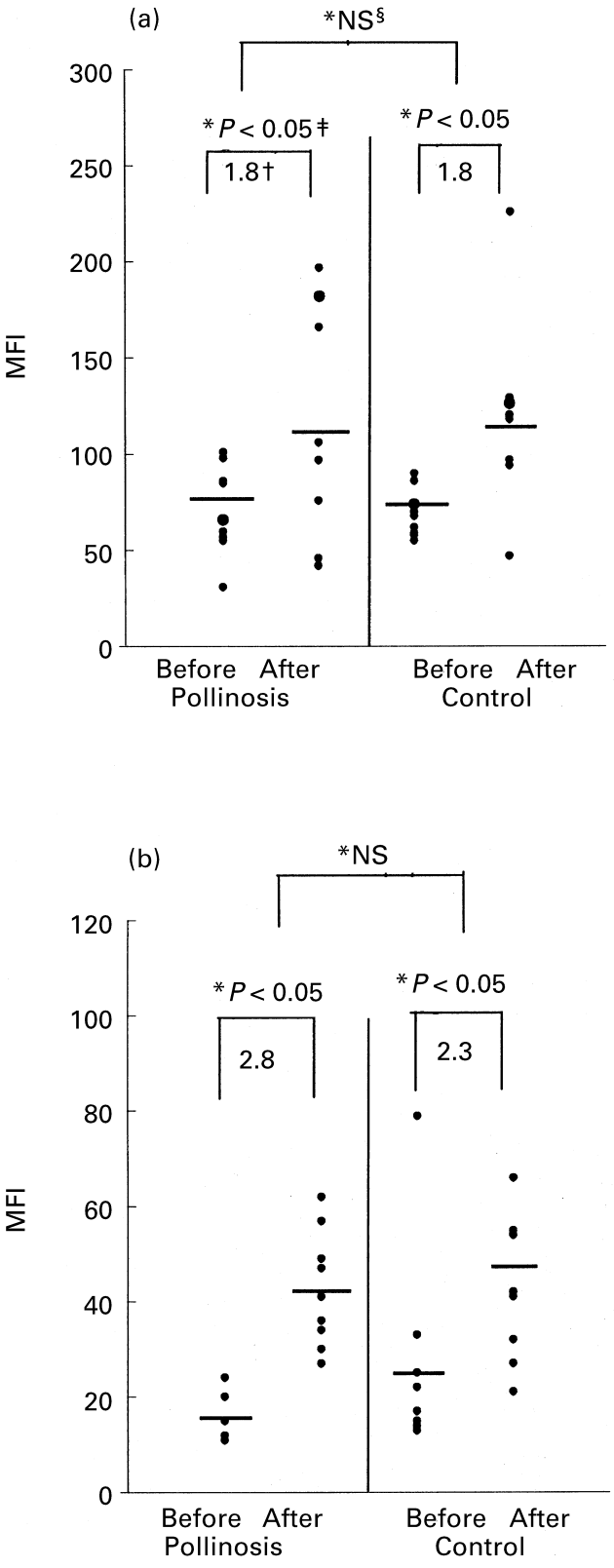
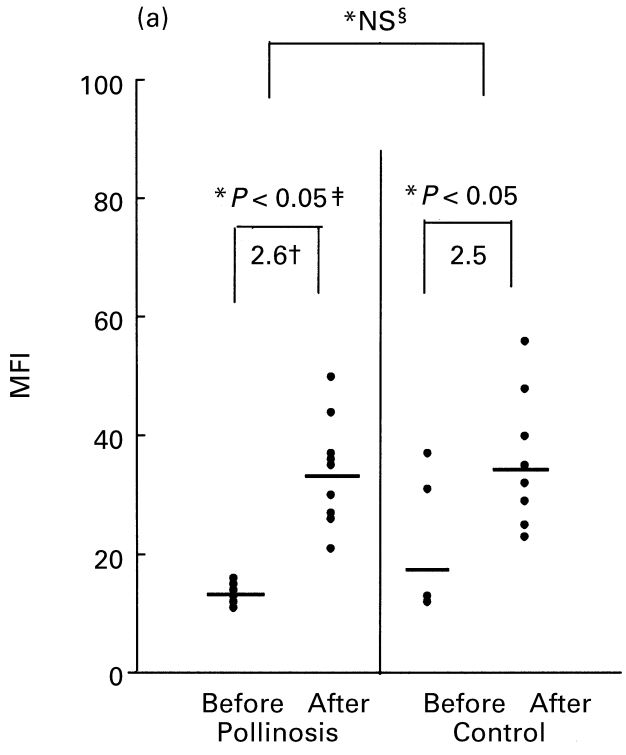
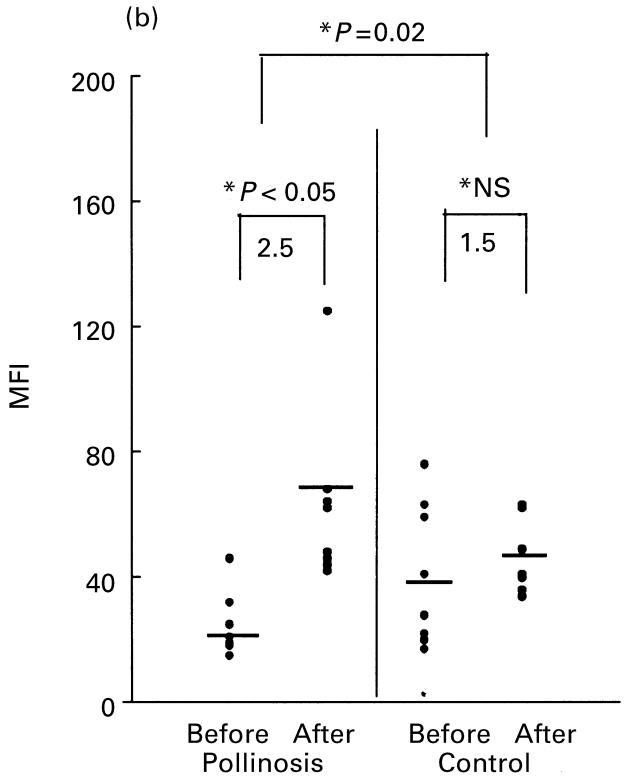


Fig. 2. (a) Expression of CD28 on $CD4^+$ cells in pollinosis subjects compared with controls after *in vitro* stimulation. The data presented are MFI before and after stimulation. (b) CD152 (CTLA-4) on $CD4^+$ cells in pollinosis subjects compared with controls after *in vitro* stimulation. The bars indicate the mean value of MFI in each group. †Upregulation ratio (MFI after stimulation/MFI before stimulation) was compared in pollinosis and control subjects. ‡MFI after stimulation was compared with MFI before stimulation in each group. §NS, not statistically significant





initiation of antigen-specific T-cell responses, upregulation of cytokines and promotion of T-cell expansion and differentiation [33]. In pollinosis subjects, CD28 molecules on $CD4^+$ T cells were not significantly upregulated after *in vitro* stimulation compared with those in control subjects. However, it has been reported that CD28-deficient mice have normal levels of IFNγ but markedly reduced levels of IL-4 *in vivo* Leishmania system [34].

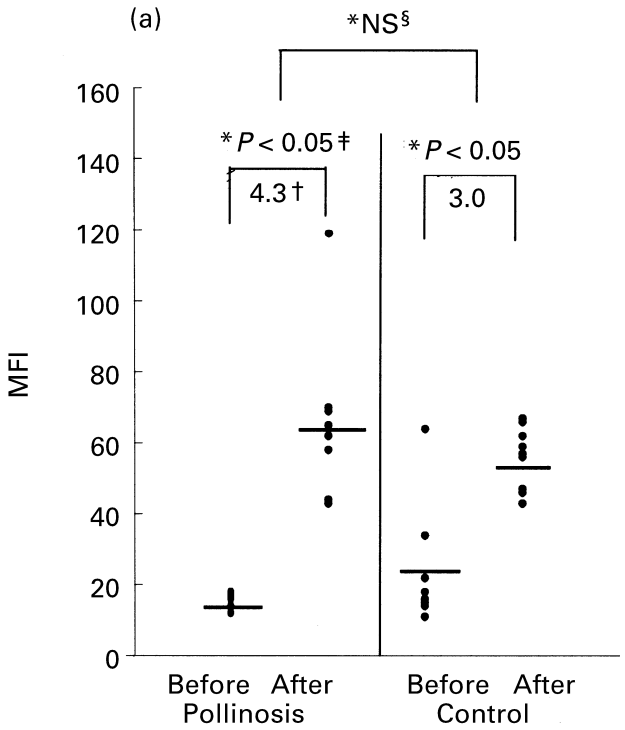
A second receptor for the B7 ligand, CTLA-4 (CD152), was also found on T cells and was upregulated on the surface of T cells after CD28-B7-costimulated T-cell activation. CTLA-4 was initially thought to be a positive costimulatory receptor similar to CD28 which functions to sustain the immune response. However, more recent data have indicated that CTLA-4 may actually function to downregulate the immune response [28]. CTLA-4-knockout mice exhibit extensive lymphoproliferative disease, showing considerable lymphocytic infiltration of many organ systems [35]. CTLA-4-Ig fusion protein could block allergen-induced proliferation and cytokine production by PBMCs from atopic allergic donors [36,37]. Anti-CTLA-4 antibodies do not induce a costimulatory signal, although they appear to enhance the costimulatory signal when used in conjunction with anti-CD28 antibodies [38]. These observations are consistent with a role of CTLA-4 as a negative regulator of T-cell proliferation. In pollinosis subjects, CTLA-4 levels were not significantly upregulated after *in vitro* stimulation compared with control subjects. However, anti-CTLA-4 antibody enhanced TH2-type cytokine production in a model of *Nippostrongyrus brasiliensis* infection [39]. Recently, it was suggested that CTLA-4 does not necessarily deliver a negative regulatory signal [40].

In pollinosis subjects, B7-1 levels were not significantly upregulated after *in vitro* stimulation compared with control subjects. However, significant upregulation of B7-2 level was observed in pollinosis subjects compared with controls, since activation of T cells was highly dependent on B7-2, in agreement with the previously published observation that B7-2 is the predominant CD28 ligand early in the immune response [19,24,41]. The level of B7-2 expression has also

Fig. 3. (a) Expression of CD80 (B7-1) on $CD19^+$ cells in pollinosis subjects compared with controls after *in vitro* stimulation. The data presented are MFI before and after stimulation. (b) Expression of CD86 (B7-2) on $CD19^+$ cells in pollinosis subjects compared with controls after *in vitro* stimulation. The data presented are MFI before and after stimulation. The bars indicate the mean value of MFI in each group. †Upregulation ratio (MFI after stimulation/MFI before stimulation) was compared in pollinosis and control subjects. ‡MFI after stimulation was compared with MFI before stimulation in each group. §NS, not statistically significant

been reported to be markedly higher than that of B7-1 during the course of primary immune responses *in vitro* [13]. There are clear differences in the timing of the expression of B7-1 and B7-2 during immune responses as well as differences in their expression on different APCs [19]. When B lymphocytes are activated by cross-linking with their surface Ig receptors or MHC class II molecules, B7-2 rapidly appears on the cell surface, followed by B7-1 after about 24–48 h [28]. Based on the relative timing of their expression, B7-2 may be the physiological ligand for CD28, while B7-1 is primarily a ligand for CTLA-4 [31]. Antibody blocking experiments suggested that B7-2 is the major CD28 ligand that initiates CD28-dependent costimulation in unprimed lymph node cells. In contrast, B7-1 costimulation appears to be upregulated later during activation of immune responses, and has been suggested to be the major costimulatory ligand present during persistent infections [28]. B7-1 and B7-2 could also serve as counter-receptors that transduce distinct signals to APCs upon engagement by CD28 or CTLA-4. The intracellular domains of B7-1 and B7-2 are distinct and could mediate transduction of different signals [28]. There are several possibilities for the differential ability of B7-1 and B7-2 to affect TH cell differentiation [28,33]. Kuchroo *et al.* have shown that blockade of B7-1 ameliorated experimental autoimmune encephalomyelitis (EAE), a TH1-mediated disease, whereas blockade of B7-2 exacerbated this disease [21]. Several investigators have demonstrated a role of B7-2 in differentiation of naive T cells into the TH2 subsets [24–26]. B7-2 rather than B7-1 may be the costimulatory molecule involved in allergen activation of PBMCs, in agreement with a previous report [42,43]. In addition, when cells obtained from the airways were examined, allergen-induced proliferation of bronchoalveolar lavage T cells from atopic asthmatic subjects was inhibited by anti-CD86 but not anti-CD80 [43].

In pollinosis subjects, CD40L (CD154) levels were not upregulated after *in vitro* stimulation compared with controls. In contrast, CD40 was upregulated after *in vitro* stimulation compared with controls. CD40–CD40L interactions control costimulatory activity on B cells by inducing



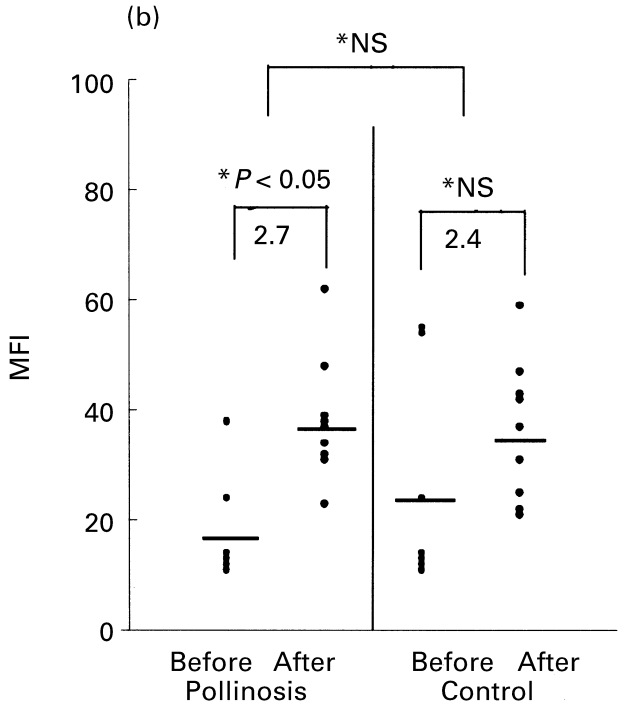


Fig. 4. (a) Expression of CD40 on $CD19^+$ cells from pollinosis subjects compared with those from controls after *in vitro* stimulation. The data presented are MFI before and after stimulation. (b) CD154 (CD40L) on $CD4^+$ cells from pollinosis subjects compared with those from controls after *in vitro* stimulation. The bars indicate the mean value of MFI in each group. †Upregulation ratio (MFI after stimulation/MFI before stimulation) was compared in pollinosis and control subjects. ‡MFI after stimulation was compared with MFI before stimulation in each group. §NS, not statistically significant

expression of B7-1, B7-2 and other molecules, and therefore might play a key role in T-cell activation and development of effector functions [44]. Recently CD40–CD40L interactions were shown to play a crucial role in the development of effector functions, as well as the newly described role in T-cell priming: CD40L is required for B-cell activation and differentiation, and to activate macrophages and monocytes to produce TNFα, IL-1, IL-12 and IFNγ [45]. CD40–CD40L interaction during T-cell activation by antigen-presenting macrophages results in IL-12 production, and IL-12 has been shown to play a critical role in development of TH1 cells [46]. Therefore, it is necessary to further investigate whether CD40–CD40L interactions could cause differentiation of T cells into TH1 and TH2.

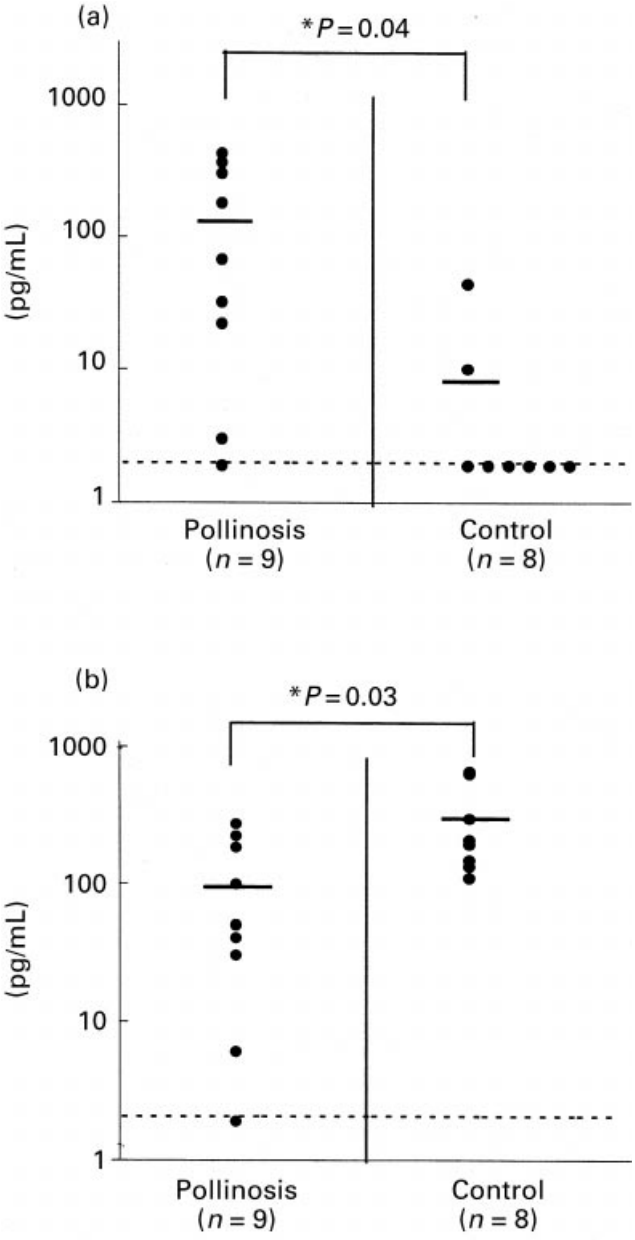


Fig. 5. Cytokine production by allergen-stimulated PBMCs measured by ELISA. (a) Production of IL-5. (b) Production of IFNγ. The bars indicate the mean value of cytokine production in each group. The broken line indicates the sensitivity of cytokine production. The sensitivities of the assays were 2 pg/mL for IL-5 and IFNγ

The TH1 phenotype is defined by T cells producing the cytokines IL-2, TNFβ and IFNγ, which activate macrophages and induce delayed-type hypersensitivity responses. TH2 cells produce IL-4, IL-5 and IL-10, which stimulate mast cells, eosinophils and the production of immunoglobulins (IgG1 and IgE) [28]. In our experiments, IL-4 was not detected in the culture supernatants obtained 6 days after *in vitro* stimulation. Our results were unexpected since a number of studies have detected high levels of IL-4 production in allergic and atopic patients [47–50]. However, several investigators have shown that IL-4 production was not detected in the culture supernatants after *in vitro* stimulation [51,52]. As the lack of detectable IL-4 production by allergen-stimulated PBMCs in our study might have been due to consumption in the culture, IL-4 production was measured in the culture supernatants obtained 1, 2 and 3 days after *in vitro* stimulation. However, IL-4 could not be detected in the culture supernatants in the early phase of culture (data not shown). It has previously been shown that detectable IL-4 production may require repeated allergen-specific T-cell stimulation [50]. Previous *in vivo* experiments have shown that exposure to low doses of antigen favours the priming of TH1 responses, while high doses of antigen favour the priming of TH2 responses. In contrast, it was reported that IL-4 production in the allergen-stimulated PBMC culture supernatants was induced at very low allergen concentrations (0.003–0.01 μg/mL), but not at high concentrations (10–30 μg/mL) [53]. We stimulated PBMCs at a concentration of 120 ng/mL of cedar-pollen antigen, Cry j 1, which was shown to be the optimal concentration for T-cell proliferation by ^{3}H-thymidine incorporation (data not shown). It is unclear why IL-4 production was not detected in our system.

The IL-5 production by allergen-stimulated PBMCs of pollinosis subjects was significantly higher than that of control subjects. In contrast, the IFNγ production by the cells of control subjects was significantly higher than that of pollinosis subjects. The production of IL-5 and IFNγ was used to determine whether the cytokine profile of the responding T-cell population reflects the allergic phenotype of the patients. The production of IL-5 by PBMCs has recently been reported to occur only in atopic patients and to be correlated with the severity of the disease, indicating that it is an excellent marker for TH2 activation in

PBMC cultures [54]. Moreover, it was recently reported that IL-5 production by PBMCs in response to allergens was completely inhibited by addition of IFNγ [55]. These findings are compatible with our observations.

In this study, we focused on B cells as APCs for immune responses in pollinosis subjects. Recently, Larché *et al.* reported that alveolar macrophages from asthmatic, but not control subjects showed APC activity to autologous T cells [43]. It is therefore necessary to further investigate the roles of other APC such as macrophages and dendritic cells in pollinosis subjects.

In conclusion, the results of this study indicated that the TH2 response is predominant in pollinosis subjects, and that $CD19^+$ cells from pollinosis subjects express B7-2 more strongly than those of controls after *in vitro* stimulation. B7-2 rather than B7-1 may be the costimulatory molecule involved in allergen activation of PBMCs.

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