

THE influence of anti-allergic drugs, epinastine hydrochloride (EP) and disodium cromoglycate (DSCG), on the co-stimulatory molecule expression was examined using *in vitro* cell culture technique. Spleen cells obtained from BALB/c mice 10 days after immunization with haemocyanin absorbed to aluminium hydroxide were cultured in the presence of 100.0 µg/ml haemocyanin and various concentrations of the agents. Low concentrations ($<1.5 \times 10^{-4}$ M) of EP and DSCG did not influence spleen cell blastic activity induced by antigenic stimulation, whereas these agents caused significant inhibition of spleen cell activation when 2×10^{-4} M of the agents were added to cell cultures. EP and DSCG also did not affect blastic activity of sensitized splenic T cells by anti-CD3 monoclonal antibody stimulation even when these cells were cultured in the presence of 2×10^{-4} M of the agents. We next examined the influence of EP and DSCG on the expression of co-stimulatory molecules on spleen cells in response to antigenic stimulation. Sensitized spleen cells were cultured in the presence of 2×10^{-4} M of the agents and the expression of molecules were examined by flow cytometer 24 h later. EP and DSCG suppressed the expression of co-stimulatory molecules, CD40 and CD80, but not CD86, on splenic B cells which were enhanced by antigenic stimulation *in vitro*.

Key words: Anti-allergic agent, Co-stimulatory molecule, Expression, Inhibition, Mouse, Spleen cell

Suppressive effects of co-stimulatory molecule expressions on mouse splenocytes by anti-allergic agents *in vitro*

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Introduction

Current theories on the pathogenesis of allergic diseases such as asthma, atopic dermatitis and allergic rhinitis, clearly show that inflammatory responses are important determinants of the diseases.¹ Several inflammatory cells including eosinophils and mast cells, and IgE antibody have been postulated to participate in the inflammatory responses to antigenic stimulation.^{2,3} It is generally accepted that the central cells in these responses are the T lymphocytes: T lymphocytes, especially helper T lymphocytes, upon contact with allergens, activate and release several types of lymphokines that control the establishment and regulation of the inflammatory responses that underly many allergic diseases.

Antigen-specific T cell activation requires triggering via the antigen-specific T cell receptor and a co-stimulatory signal usually provided by antigen presenting cells (APC) including B cells and macrophages.^{4–7} Interaction of T cells with antigen in the absence of sufficient co-stimulation can result in T cell unresponsiveness—termed anergy.^{7,8} A number of APC surface determinants have been shown to be capable of providing T cell co-stimulation. Activated APC

express CD80, which co-stimulates T cells via binding to the counter receptor CD28.^{4,7} CD86, which also binds to CD28, is constitutively expressed on professional and non-professional APC earlier than CD80.^{4,7} The interaction between CD40 molecules and its ligand, CD40L, on T cells has also been identified as an important co-stimulatory signal required for switch recombination to IgE synthesis.^{9,10}

A number of anti-allergic drugs such as azelastine, epinastine hydrochloride (EP), and disodium cromoglycate (DSCG) have been developed and are used clinically in the treatment and the management of allergic diseases. The mechanisms of action of these agents on allergic diseases are generally believed to be owing, in part, to their inhibitory action on chemical mediator release from effector cells such as eosinophils and mast cells.^{11,12} Although these agents are also reported to display antagonistic effects on chemical mediators,^{13,14} there is little information about the influence of anti-allergic agents on co-stimulatory molecule expression. Therefore, in this study we examined whether anti-allergic agents could modulate the expression of co-stimulatory molecules on lymphocytes by an *in vitro* cell culture technique.

Materials and methods

Mice

Specific pathogen free, male BALB/c mice, 5 weeks of age were purchased from Charles River Japan Inc. (Atsugi, Japan). Each experimental and control group consisted of five mice. Animal care and handling were in accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals (Prime Minister's Office of Japan, Publication No. 85-23, 1985). The experimental protocols of this study were approved by the Animal Care and Use Committee of Showa University.

Immunization

Mice were immunized by intraperitoneal injection of 8.0 µg/ml haemocyanin (Sigma Chemical Co., St Louis, MO) adsorbed to 4.0 mg aluminium hydroxide (Wako Chemical Co., Ltd, Osaka, Japan) in 0.5 ml saline.

Agents

EP was kindly supplied by Boehringer Ingelheim (Ingelheim, Germany) as a water soluble pure powder. The powder was dissolved in saline at a concentration of 5×10^{-2} M. This solution was then filtered through a 0.22 µm filter (Nihon Millipore Co., Ltd, Yonezawa, Japan) and stored as a stock solution at 4°C until used. All dilutions used in this study were prepared from this stock solution by dilution with RPMI-1640 medium (Wako Chemical) supplemented with 10% foetal calf serum (Sigma chemical; RPMI-FCS). DSCG was purchased from Fujisawa-Fison Co., Osaka, Japan as a solution in distilled water. This solution contained 5.0 M DSCG. All dilutions used in this study were prepared from this solution.

Cell preparation

Spleen was removed from five individual mice 10 days after the immunization. These organs were pressed through 60 gauge steel sieves into saline. The cells were washed once with saline and then treated with Tris-buffered ammonium chloride (17 mM Tris-HCl-0.73% NH₄Cl, pH 7.6) for 5 min to lyse red blood cells. After passing through 200 gauge steel sieves, the residual cells were washed three times, resuspended in saline at a concentration of 1×10^6 cells/ml and used for splenocytes. In the case of experiments using purified T cells, T cells were separated from splenocytes using a magnetic cell separator (Milteny Biotec GmbH, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Splenocytes were labeled with anti-mouse CD19 monoclonal antibody (mAb)-coated magnetic microbeads (Milteny Biotec GmbH). These cells were then applied to the column placed in the separator and the column was washed with PBS. The entire effluated cells were washed

twice and resuspended in saline at a concentration of 1×10^6 cells/ml. The purity of the cells was checked by incubating separated cells with fluorescein isothiocyanate (FITC)-conjugated mAb to CD90 (PharMingen, San Diego, CA). Fluorescence microscopic examination revealed that more than 98% of the cells were CD90 positive, and these cells were used as splenic T cells.

Cell proliferation assay

Splenocytes prepared as above were washed once and resuspended in RPMI-1640 medium (Wako Chemical) supplemented with 10% foetal calf serum (Sigma Chemical; RPMI-FCS) at a concentration of 1×10^6 cells/ml. To examine blastic activity of the cells, 100 µl of cell suspension was introduced into each well of 96-well flat-bottomed culture plates (Nunc, Denmark). Various concentrations of the anti-allergic agents, EP and DSCG, and 100.0 µg/ml haemocyanin were added in another 100 µl to give a total volume of 200 µl. These mixtures were then incubated at 37°C in a humidified atmosphere of 5% CO₂ in air. After 40 h culture, 37 kBq of ³H-thymidine (specific activity 740 GBq/mmol, New England Nuclear, Boston, MA, USA) was added to each well, and the plate was maintained for another 8 h. Cells were collected on glass fiber filters for liquid scintillation counting using a Packard Tricarb liquid scintillation spectrometer. The results were expressed as mean counts per minute (cpm) ± SD of triplicate cultures. For examination of the proliferative response of splenocytes to anti-CD3 stimulation, 100 µl of PBS containing 20.0 µg/ml of mAb to mouse CD3ε (Genzyme, Minneapolis, MN, USA) was introduced into each well of 96-well flat-bottomed culture plates (Nunc, Denmark) in triplicate and incubated overnight at 4°C.¹⁵ Before use, the plates were washed once with 200 µl PBS. Splenic T cells prepared as above were cultured in mAb-coated plates that contained various concentrations of agents at 2×10^5 cells in a final volume of 200 µl for 48 h. The cell proliferation was assessed in a similar manner. For examination of the influence of the agents on co-stimulatory molecule expressions, 0.5 ml of the cell suspensions (5×10^5 cells) were dispensed into 24-well flat-bottomed culture plates (NUNC) that contained 0.5 ml of various concentrations of the agents and 100.0 µg/ml antigen. The plates were maintained for 24 h at 37°C in a humidified atmosphere of 5% CO₂ in air. The cells were then collected by centrifugation, washed twice with saline, resuspended at a concentration of 1×10^5 cells/ml, and stored on ice until processed.

Flow cytometry

The mAbs used for flow cytometry were anti-mouse CD16/32 mAb, FITC-conjugated anti-mouse CD40, CD80 and CD86 mAbs. They were purchased from

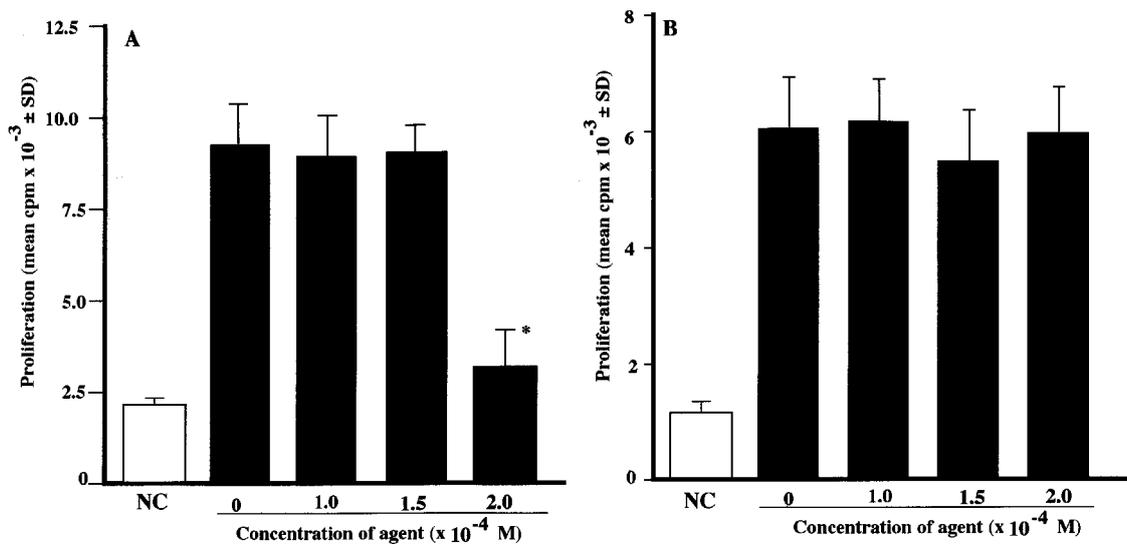


FIG. 1. Influence of epinastine hydrochloride (EP) on *in vitro* proliferative response of splenic lymphocytes. BALB/c mice were immunized by intraperitoneal injection with 8.0 µg haemocyanin adsorbed to 4.0 mg aluminium hydroxide in a volume of 0.5 ml. Spleen cells (1×10^5 cells/0.1 ml) prepared from mice 10 days later were cultured in the presence of various concentrations of EP for 48 h, which were stimulated with specific antigen (A). Splenic T cells (2×10^5 cells/0.1 ml) were also cultured with immobilized anti-CD3 ϵ monoclonal antibody in the presence of various concentrations of EP for 48 h (B). These cells were pulsed with 1.0 µCi ³H-thymidine for the last 8 h of culture. The results were expressed as the mean ± SD of five individual mice. NC: non-stimulated control. * $P < 0.01$, significantly different as compared with no agent.

PharMingen. Before staining CD40, CD80 and CD86 molecules with specific antibodies, the purified cultured B cells (1×10^6 cells) were treated with 1.0 µg of anti-mouse CD16/32 mAb for 5 min at 4°C to block non-specific Fc receptor mediated binding of antibodies. Pre-treated cells were then stained with either FITC-conjugated mAb to CD40, CD80 or CD86 for 25 min at 25°C. After washing once, cells were suspended in saline, and assayed for fluorescence intensity on cells using Flow cytometer (Becton Dickinson, Mountain View, CA, USA).

Statistical analysis

The statistical significance of the difference in the mean value between two groups was examined by the Mann–Whitney *U* test.

Results

Influence of EP on proliferative response of lymphocytes *in vitro*

The first set of experiments was designed to examine the influence of EP on proliferative response of spleen cells induced by *in vitro* stimulation with antigens. Spleen cells prepared from five individual mice immunized with haemocyanin were cultured *in vitro* in the presence of 100.0 µg/ml haemocyanin and various concentrations of EP for 48 h. As shown in Fig. 1A, low doses of EP (10^{-4} M and 1.5×10^{-4} M) did not affect the proliferative activities of spleen cells in response to haemocyanin stimulation *in vitro*. How-

ever, a statistically significant inhibition of cell proliferation was observed when the cells were cultured in the presence of 2×10^{-4} M of the agent. Further experiments were carried out to examine whether the suppressive activity of EP on antigen-induced cell proliferation was also observed in cells stimulated by anti-CD3 mAb. To do this, splenic T cells from haemocyanin-immunized mice were cultured with solid phase of mAb in the presence of various doses of EP for 48 h. As shown in Fig. 1B, EP did not interfere with the proliferative response of sensitized splenic T cells even when the cells were cultured with 2×10^{-4} M EP.

Influence of EP on co-stimulatory molecule expression

This study was carried out to examine the influence of EP on co-stimulatory molecule expressions on splenocytes. Spleen cells prepared from haemocyanin-immunized mice were cultured *in vitro* in the presence of 100.0 µg/ml haemocyanin and 2×10^{-4} M of EP for 24 h. The cultured cells were stained with FITC-labeled mAbs against co-stimulatory molecules and were analyzed by flow cytometry for the expression of CD40, CD80 and CD86 on their cell surface. As shown in Fig. 2, addition of EP into cell cultures at a dose of 2×10^{-4} M significantly suppressed CD40 expression on the cells, which were enhanced by *in vitro* stimulation with haemocyanin. EP also suppresses the ability of the cells to express CD80 molecules on their cell surface induced by antigenic stimulation *in vitro* (Fig. 2).

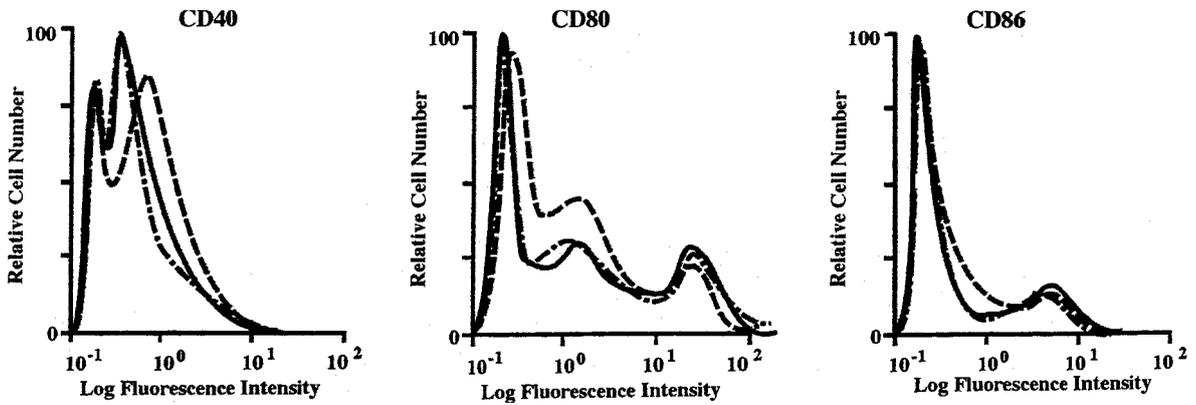


FIG. 2. Influence of epinastine hydrochloride (EP) on co-stimulatory molecule expression on spleen cells induced by antigenic stimulation. Spleen cells (1×10^6 cells/ml) prepared from five individual mice 10 days after immunization were cultured with or without 2×10^{-4} M EP for 24 h. The expression was analyzed by flow cytometer: (—) cultured without antigen; (---) cultured with antigen; (- · - · -) cultured with antigen and EP.

Influence of DSCG on the response of splenocytes to antigenic stimulation

Since many kinds of anti-allergic agents are used clinically, the results described above leave open the questions of whether these anti-allergic agents have similar suppressive effects on cell functions to those observed in EP. Therefore, we chose DSCG, one of the best-known anti-allergic agents, and examined whether it also has inhibitory effects on cell functions, such as proliferation and co-stimulatory molecule expression, induced by *in vitro* antigenic stimulation. As in the case of EP, DSCG exerted suppressive effects on antigen-induced, but not anti-CD3-mediated, proliferative responses of splenocytes

(Fig. 3A and B), when the cells were cultured in the presence of 2×10^{-4} M DSCG. Flow cytometric analysis also revealed the suppressive activity of DSCG on the expression of co-stimulatory molecules, CD40 and CD80, which are enhanced by antigenic stimulation *in vitro* (Fig. 4)

Discussion

Asthma and atopic allergy are characterized by infiltration of the bronchial and nasal mucosa with large numbers of activated eosinophils and mast cells and the presence of elevated concentrations of chemical mediators as well as granule-derived basic proteins.^{2,3} The acute symptoms of allergy and asthma

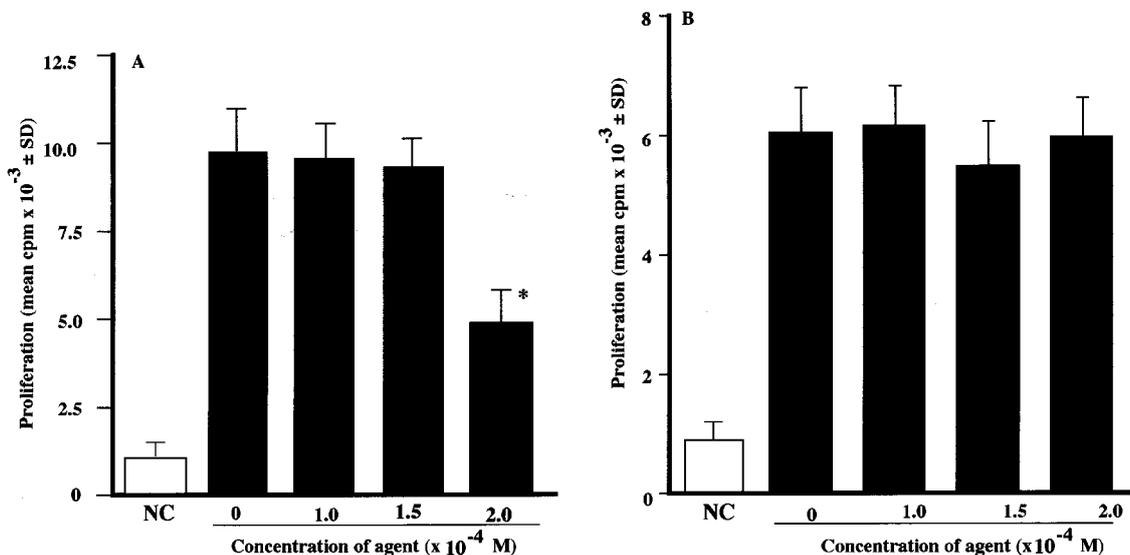


FIG. 3. Influence of disodium cromoglycate (DSCG) on proliferative response of lymphocytes *in vitro*. BALB/c mice were immunized by intraperitoneal injection with 8.0 μ g haemocyanin absorbed to 4.0 mg aluminium hydroxide in a volume of 0.5 ml. Spleen cells (1×10^5 cells/0.1 ml) prepared from mice 10 days later were cultured in the presence of various concentrations of DSCG for 48 h, which were stimulated with specific antigen (A). Splenic T cells were also cultured with immobilized anti-CD3 ϵ monoclonal antibody in the presence of various concentrations of DSCG for 48 h (B). These cells were pulsed with 1.0 μ Ci 3 H-thymidine for the last 8 h of culture. The results were expressed as the mean \pm SD of five individual mice. NC: non-stimulated control. * $P < 0.05$, significantly different as compared with no agent.

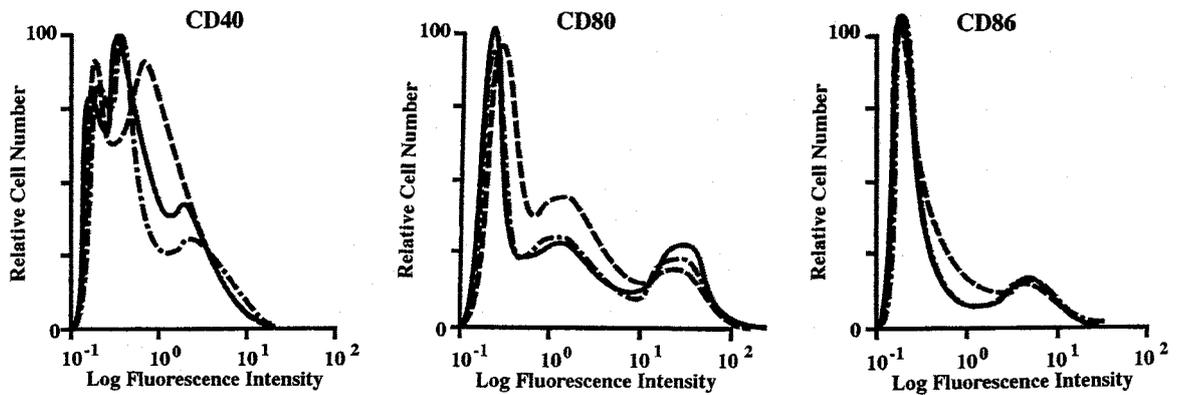


FIG. 4. Influence of disodium cromoglycate (DSCG) on co-stimulatory molecule expression on spleen cells induced by antigenic stimulation. Spleen cells (1×10^6 cells/ml) prepared from five individual mice 10 days after immunization were cultured with or without 2×10^{-4} M of DSCG for 24 h. The expression was analyzed by flow cytometer: (—) cultured without antigen; (- - -) cultured with antigen; (- · - ·) cultured with antigen and DSCG.

such as sneezing, bronchospasm and hives are believed to be largely the results of mediator release from mast cells and basophils,^{2,3} whereas chronic symptoms, the result of allergic inflammation, can be explained on the basis of eosinophil-mediated tissue damage.^{2,3,16} Based on these established ideas, the treatment of allergic diseases still focuses on the inhibition of mediator release or on blocking the binding of soluble mediators to their receptors. However, additional evidence clearly shows that T cells play a central role in the driving and maintenance of all these processes by elaboration of several types of cytokines.^{2,3,16,17} There is now also circumstantial evidence that the CD28/B7 co-stimulatory is critical in T cell activation, proliferation and cytokine production.^{4-7,18} Yet the action of anti-allergic agents on the co-stimulatory pathway is poorly understood. To examine the influence of anti-allergic agents in the co-stimulatory pathway, it was first tested on the response of lymphocytes to *in vitro* antigenic stimulation by examining DNA synthetic activity. The present results (Figs 1A and 3A) clearly show the inhibitory effect of EP and DSCG on DNA synthetic activity (proliferation) of sensitized spleen cells in response to *in vitro* stimulation with specific antigen. However, EP and DSCG could not inhibit DNA synthetic activity of sensitized splenic T cells by anti-CD3 stimulation (Figs 1B and 3B), suggesting that inhibition of the proliferative responses of sensitized spleen cells by specific antigen is not due to cytotoxic action of the agents to splenic T cells. It is reported that anti-allergic agents did not interfere with the ability of APC to process antigens.¹⁹ The present results, therefore, may suggest that EP and DSCG inhibit proliferation of sensitized spleen cells induced by antigenic stimulation through the suppression of signal transmission required for DNA synthesis.

T and B cells play a critical role in the pathogenesis of allergic diseases. Activation of resting lymphocytes requires the engagement of the T cell receptor with a

peptide/MHC as well as the engagement of appropriate co-stimulatory molecules.⁴⁻⁷ In particular, signals through the CD28/CTLA-4 co-stimulatory pathway are essential for primary activation of antigen specific T cells.²⁰ It is reported that CTLA-4 immunoglobulin fusion protein (CTLA-4Ig), a blocker of CD28/B7 co-stimulation, could block allergen-induced proliferation and cytokine production of peripheral blood mononuclear cells from atopic donors, when the cells were cultured *in vitro* in the presence of CTLA-4Ig.¹⁸ In addition to the CD28/CTLA-4 co-stimulatory signals, CD40 ligand (CD40L) was also reported to play an important role in the interaction between helper T cells and APC.^{21,22} The CD40L-CD40 interaction can upregulate the expression of B7 molecules on APC, which are essential for helper T cell activation, and enhances T cell proliferative responses.^{23,24} Therefore, we examined whether EP and DSCG suppress the expression of co-stimulatory molecules, CD40, CD80 and CD86, on sensitized B lymphocytes, induced by *in vitro* stimulation with specific antigen and resulted in inhibition of *in vitro* T cell proliferative responses. The present results clearly show that EP and DSCG could suppress the expression of co-stimulatory molecules, CD40 and CD80 but not CD86, on sensitized B cells which were enhanced by *in vitro* stimulation with antigen (Figs. 2 and 4). Prophylactic treatment of the patients with anti-allergic agents is recognized to prevent the development of the diseases by inhibition of IgE hyper-production,^{9,25} but the mechanisms are not well defined. Induction of IgE synthesis from B cells requires two distinct signals. The first signal is delivered by IL-4 and the second is provided by T-B cell contact through the ligation of CD40 and CD40L.^{9,10} Together with the present results, it may be speculated that anti-allergic agents exerted inhibitory effects on the development of the diseases through the suppression of CD40 expression, when agents were administered prophylactically.

Recent reports have examined the co-stimulatory dependence of allergen-specific human T cells, and the role of these CD28 and CD80 in animal models of allergic inflammation. De Boer *et al.* showed that CD80 could act as a co-stimulator molecule for Th2 type cytokine, IL-4 and IL-5, productions from human peripheral blood T cells activated by mAb directed at T cell receptor.²⁶ It is also reported that CD80 blockade prevents antigen specific proliferative responses of T cells from atopic dermatitis against *Dermaphagoides farinae*.²⁷ In murine models for inflammatory diseases, blockade of CD80 is reported to ameliorate the diseases.²⁸ With regard to the influence of CD80 on effector cell (e.g. eosinophil) functions, Tsuyuki *et al.* showed that intranasal administration of anti-CD80 mAb inhibited eosinophil accumulation in lungs and airway tracts, but was less effective in inhibiting peripheral blood eosinophilia induced by aerosol provocation of allergen.²⁹ CD80 blockade was also reported to be able to prevent eosinophil influx into lungs and airway tracts when the mice were injected with anti-CD80 mAb during the aerosol challenge.³⁰ These suppressive effects of CD80 may be owing to its inhibitory action on adhesion molecule expressions, since CD80 blockade is reported to prevent acquisition by lymphocytes including eosinophils of the adhesion molecules involved in recirculation through tissue, and upregulation of their ligands such as ICAM-1 on endothelium.³¹ Judging from these reports, the present results showing the suppressive effect of anti-allergic agents on CD80, but not CD86, expression suggest that anti-allergic agents, especially EP and DSCG, are effective in the prevention of allergic inflammatory responses in lungs and airway tracts.

Pharmacological studies revealed that EP and DSCG prevent calcium cation influx into immune cells,^{14,32} resulting in reduced permeability of cell membrane and inhibition of expression of proteins synthesized in the cytosol. It is also reported that anti-allergic agents including EP and DSCG increase intracellular cyclic adenosine monophosphate (cAMP) level by inhibiting adenylate cyclase activity.^{14,33} Elevation of intracellular cAMP level elaborates an important down-regulatory signal in the release of proteins synthesized in the cytosol.^{34,35} From these reports, it is possible that EP and DSCG cause prevention of the accumulation of calcium cation in the cytosol and enhance intracellular cAMP level resulting in inhibition of CD molecule expressions. Although the present data add novel information about the beneficial effects of anti-allergic agents on the diseases, the precise mechanism(s) by which the agents inhibit co-stimulatory molecule expressions is not yet fully understood. Further experiments are needed to clarify this point.

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Accepted 2 May 2000



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