Antigen-specific Enhancements of CD80 mRNA Expression in Experimentally Sensitized Dogs with Japanese Cedar Pollen

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ABSTRACT. CD80, CD86, CD28 and Cytotoxic T lymphocyte antigen-4 (CTLA-4) are well-known co-stimulatory molecules that form the major co-stimulatory pathway essential for full activation of T cells. To investigate their role in pathogenesis of immune-mediated diseases, 12 dogs were sensitized experimentally to Japanese cedar pollen antigen (CPAg) as models of allergic diseases in dogs. After sensitization, lymphocyte stimulation test (LST) was carried out to evaluate reactivity to CPAg, and semi-quantitative real-time RT-PCR analysis of CPG-stimulated peripheral blood mononuclear cells (PBMCs) to evaluate the expression of co-stimulatory molecules. As a result, CPG-specific enhancements of CD80 expression were detected in all sensitized dogs. Furthermore, two different kinetics of its enhancements according to the blastogenic responses to CPG were also observed. Expression of CD28, CTLA-4 and CD86 were suppressed following CPG-stimulation. The result of the present study indicated the potential role of the CD28-CD80 co-stimulation pathway in pathogenesis of allergic diseases in dogs.

KEY WORDS: allergic disease, canine, CD80, co-stimulatory molecule, quantitation.

It is known that co-stimulatory signals are essential for full activation of T cells in addition to recognition of antigen presented by antigen presenting cells (APCs). This signal is transmitted through interactions of adhesion molecules called “co-stimulatory molecules” [4, 10]. To date, many molecules are known to “co-stimulate” T cells, and affect their proliferation, production of cytokines and induction of other adhesion molecules during antigen-specific immune responses.

Among co-stimulatory molecules, B7-CD28/CTLA-4 pathway are the best characterized in humans and mice, but are rarely reported in veterinary medicine. This pathway includes CD28 and cytotoxic T lymphocyte antigen-4 (CTLA-4) expressed on T cells [3, 7], and CD80 (B7–1) and CD86 (B7–2) expressed on APCs, B cells and activated T cells [2, 6]. Co-stimulation through interactions between CD28 and CD80/86 following antigen recognition activate antigen-specific T cell populations, and induce further immune-responses, whereas interactions between CTLA-4 and CD80/86 provide negative signals and halt activity of T cells [1, 12].

Based on the importance of those co-stimulatory molecules in immune responses, it is assumed that T cell activation through the co-stimulatory pathway is involved even in disease states. Especially, it is expected that abnormality or deviation in expression of co-stimulatory molecules could play an important role in the pathogenic processes of immune-mediated diseases because variety of T cell activities including production of cytokines and chemokines are affected by co-stimulation through this pathway [8, 13]. In this study, we generated experimentally sensitized dogs as models of allergic diseases and examined the expression of B7-CD28/CTLA-4 molecules in PBMCs by real-time RT-PCR analysis. There are few studies reporting fluctuations in the expression of co-stimulatory molecules following antigen stimulation in vivo, and the analyses performed here may show participation of co-stimulatory molecules in the pathogenic process of allergic diseases.

MATERIALS AND METHODS

Experimental sensitization of dogs: Twelve intact beagles, aged 4 to 5 months, were sensitized to Japanese cedar (Cryptomeria japonica, C. japonica) pollen antigen (CPAg) [19] as described previously [18]. Briefly, dogs were injected with 100 µg CPG conjugated with 20 mg alum subcutaneously for each injection. Sensitization was performed twice at two weeks intervals, and dogs were boosted with 500 µg CPG two weeks after the last injection. Whole blood samples were taken 4 days after the booster injection.

Fluorometric ELISA for specific IgE to C. japonica pollen antigen: A CPG-specific serum IgE titer was determined by fluorometric ELISA as described previously [15]. Briefly, a microplate (Immulon 2, Dynatech, Chantilly, VA) was coated with 10 µg/ml CPG at 4°C overnight. Following washing, diluted sera of sensitized dogs were added to each well and incubated at room temperature (RT) for 3 hr. The plate was incubated with 0.5 µg/ml mouse anti-dog IgE monoclonal antibody [5] at 4°C overnight, and then biotinylated rat anti-mouse IgG1 monoclonal antibody (Zymed Laboratories, San Francisco, CA) at RT for 1 hr. Finally, the plate was developed by incubation with β-D-galactosidase conjugated streptavidin (Zymed Laboratories, San Francisco, CA) at RT for 1 hr, and 0.1 mM 4-methylumbelliferyl-β-D-galactoside (Sigma Chemical Co., St. Louis, MO) at 37°C for 2 hr. The fluorescence intensity was measured by a fluorescence reader (Fluoroskan, Flow Laboratories, McLean, VA) and expressed as fluorescence units.
The IgE level specific to CPAg was expressed in arbitrary units (U/ml), and calculated from a standard titration curve of a pooled serum.

**PBMC preparation:** Heparinized whole blood samples were obtained from experimentally sensitized dogs. Each sample was diluted with an equal volume of PBS and layered on Ficoll-Hypaque (Nycomed Pharma AS, Oslo, Norway). Following centrifugation at 350 x g for 40 min at RT, a layer of PBMC was collected and washed twice with PBS. The cells were resuspended in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 5% dog serum, 100 U/ml penicillin and 100 µg/ml streptomycin at a cell count of 2.0 x 10^6/ml. Each PBMC suspension was used in both lymphocyte stimulation test and antigen-stimulation.

**Lymphocyte stimulation test:** Lymphocyte stimulation test (LST) was performed as described previously [14]. Briefly, PBMC (2 x 10^6/ml) prepared as described above were placed in each well of 96-well microtiter plate and incubated at 37°C for 72 hr under stimulation with 5 µg/ml CPAg. Similarly prepared PBMC derived from healthy, non-sensitized dog was used as controls. After incubation with 1 µCi/well ^3^H-thymidine for the final 18 hr, incorporated radioactivity (cpm) was measured by a liquid scintillation counter. All samples were subjected to triplicate examinations, and reactivity to CPAg was expressed as Stimulation index (SI), the ratio of mean cpm of CPAg stimulated culture to that of unstimulated culture.

**RNA extraction:** The PBMC suspensions prepared as described above were assigned to each well of 6-well cell cultivation and stored as pre-stimulation control. The cells levels in antigen-stimulated PBMC to that of non-stimulated PBMC of the same dog for each co-stimulatory molecule were determined twice, and levels of expression enhancement following antigen-stimulation were expressed as the ratio of expression levels in antigen-stimulated PBMC to that of non-stimulated PBMC of the same dog for each co-stimulatory molecule.

**Statistical analysis:** Statistical analyses to examine variance between two groups of dogs were performed on the

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**Table 1. Sequence of primers and probes for quantitative real-time RT-PCR for canine co-stimulatory molecules and β-actin**

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer (5'→3')</th>
<th>Probe Probe sequence (5'→3')</th>
<th>Probe sequence (5'→3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD28</td>
<td>5C28Tq CCCCCAGCTTGTTGATACAAACA GCCCGGAACCTCCCTTGGAGA</td>
<td>TqprC28 TGGAGGTAATCTGAGCTGCAAGATATACCTACCAACC</td>
<td></td>
</tr>
<tr>
<td>CTLA-4</td>
<td>5CCTTq GTCTGTTGCGGCACATACAGGC CGCGGTTGAGTAGAAATCA</td>
<td>TqprCCT TGGAGGATGTTGGGCCTTTGCTG</td>
<td></td>
</tr>
<tr>
<td>CD80</td>
<td>5C80Tq GCAGCAAGACCATGTTACACCCCA GAGGGAGCCAGACCTTGA</td>
<td>TqprC80 TGGAGAACCACACCACACATCACACACACCATAT</td>
<td></td>
</tr>
<tr>
<td>CD86</td>
<td>5C86Tq CGAAACCACACCCTTGATGGCA CACAAATGACCCAACATTACAGGA</td>
<td>TqprC86 AGACCCACATCCCTGGAGGTTGCC</td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>β-ac1s TGACCTGGAATAGCCACCATTG GGTGAGAGGTGTTGAGCACAG</td>
<td>Tqprb-ac ATCGTCAACCAACTGGGACAGCAG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>β-ac1r TGACCTGGAATAGCCACCATTG GGTGAGAGGTGTTGAGCACAG</td>
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results of semi-quantitative real-time RT-PCR analysis. About all molecules analyzed in this study, results of two groups of dogs were compared using the two-sample t test. P values less than 0.05 were considered as significant.

RESULTS

Experimental sensitization of dogs with C. japonica pollen antigen: Twelve intact beagles were sensitized to CPAg as described previously [18]. Two weeks after the second injection of CPAg-conjugated alum, increase of CPAg-specific serum IgE titer had been detected in all dogs by fluorometric ELISA, confirming sensitization of these dogs to CPAg (data not shown). No clinical symptoms indicating Japanese cedar pollinosis including rhinitis, conjunctivitis or atopic dermatitis were observed in the 12 sensitized dogs during the study period as well as those of previous study [18].

Lymphocytic blastgenic responses of CPAg-stimulated PBMC of sensitized dogs: LST using PBMC of sensitized dogs was performed to evaluate the reactivity to antigen. When stimulated with CPAg, PBMCs from 6 of 12 sensitized dogs showed significant blastgenic responses indicated by [H]-thymidine incorporation of more than twice that of non-stimulated controls (SI ≥ 2.0) (Fig. 1). The responses of PBMCs from the remaining 6 dogs were less than twice that of non-stimulated controls (SI < 2.0).

Semi-quantitative real-time RT-PCR analysis for detection of co-stimulatory molecule expression in antigen-stimulated PBMC of sensitized dogs: To evaluate fluctuations in the expression of co-stimulatory molecules following stimulation with a specific antigen, we performed real-time RT-PCR analysis to quantitate the mRNA of co-stimulatory molecules in CPAg-stimulated PBMC of sensitized dogs. PBMCs obtained from these sensitized dogs and three healthy non-sensitized dogs were cultured with CPAg for 24 and 48 hr, and RNAs isolated from each culture had been subjected to the analyses.

As a result, remarkable enhancements of CD80 expression were detected in sensitized dogs following CPAg-stimulation, and mean ratios of expression enhancements were significantly higher than control dogs over 48 hr of incubation (Fig. 2). As shown in Fig. 3, mean ratio of enhancements of CD80 expression in 6 sensitized dogs that had high SI in LST (SI ≥ 2.0; high responder group) was low at 24 hr of incubation, but increased up to about 3.0 significantly higher than control dogs at 48 hr of incubation (P=0.008). On the contrary, mean ratio of enhancements of CD80 expression in 6 sensitized dogs that had low SI in LST (SI < 2.0; low responder group) was significantly higher than control dogs at 24 hr of incubation (P=0.011), but decreased down to as much as control dogs at 48 hr of incubation. Mean ratios of enhancements of CD80 expression of both groups were statistically different at both 24 and 48 hr of incubation, although peak levels during 48 hr of incubation were the same (data not shown).

Mean ratio of enhancements of CD86 expression of sensitized dogs was as much as that of control dogs at 24 hr of incubation (Fig. 4.A). However, CD86 expressions of sensitized dogs decreased and mean ratio of expression...
enhancements was significantly lower than control dogs at 48 hr of incubation (Fig. 4.B). No differences in CD86 expressions between high and low responder groups were found (data not shown).

Likewise, levels of CD28 and CTLA-4 expression of all sensitized dogs showed remarkable decrease following CPAg-stimulation, and were significantly lower than that of control dogs, which slightly decreased over 48 hr of incubation (Figs. 5 and 6; representative results are shown). No differences in CD28 and CTLA-4 expressions between high and low responder groups were found.

DISCUSSION

In this study, we generated CPAg-sensitized dogs as experimental models of allergic diseases, and examined serial changes in the expression of co-stimulatory molecules in antigen-stimulated PBMC. Among 4 molecules analyzed, only CD80 expressions were significantly enhanced by CPAg-stimulation in PBMCs of sensitized dogs, but not in control dogs. Interestingly, two distinct patterns for enhancement of CD80 expression according to the blastogenic responses to CPAg were found in the results of sensitized dogs. Enhancements of CD80 expression in
PBMCs of high responder group were low at 24 hr of incubation, but were sustained to increase over 48 hr of incubation. Conversely, enhancements of CD80 expression in PBMCs of low responder group were significantly higher than control and high responder group at 24 hr of incubation, but these enhancements were transient and decreased at 48 hr of incubation. As indicated in Fig. 3, although high and low responder groups had opposite results in LST, magnitudes of peak enhancements of CD80 expression during 48 hr of incubation were the same in both groups. Therefore it was considered that kinetics of CD80 expression, not expression level, may influence later activation of specific T cells as detected in LST. Although the mechanism for halted enhancements of CD80 expression in low responder group was unknown, distinct regulatory factors, such as another co-stimulatory pathway or soluble factors (e.g. cytokines, chemokines), may have influenced.

Compared to CD80, CD86 expressions in PBMC of sensitized dogs were suppressed following CPAg-stimulation over 48 hr of incubation, although a few dogs showed slight enhancement at 24 hr of incubation. CD86 is the functional homologue to CD80, and its expression was expected to be enhanced in CPAg-stimulated PBMCs of sensitized dogs. As CD86 expressions of control dogs did not affected by CPAg-stimulation, the expression of CD86 may be suppressed by antigen-specific fashion in experimentally sensitized dogs. In recent reports, enhanced expression of CD86 was detected in atopic patients, and Langerhans cells and B cells were shown to express this molecule [9, 16]. The expression of CD86 in PBMCs of sensitized dogs was also suppressed following CPAg-stimulation, in spite of the stimulation-enhancing character of these molecules [10]. One possible explanation for these observations is that only T cells, the restricted population of PBMC, express these molecules. It is reasonable to consider that results of CD28 and CTLA-4 may indicate failure to detect their exact fluctuations due to the minority of CPAg-specific T cells, rather than suppression, because the results of control dogs also showed decrease in expression of these molecules.

In conclusion, the results of the present study indicate that antigen presentation and activation of T cells in experimentally sensitized dogs were “co-stimulated” by CD28-CD80 interactions. Participation of CD28-CD80 interactions in allergen-specific immune responses suggests the potential for developing novel immunotherapies focused on this costimulatory pathway. For instance, induction of anergy by inhibition of the CD28-CD80 interaction, or desensitization using a CTLA-4-fused recombinant allergen that is designed to be efficiently delivered to APCs, will be useful alternatives to current therapeutic options.

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REFERENCES


