FULL PAPER *Clinical Pathology*

CTLA-4 Recombinant Protein Genetically Fused to Canine Fcε Receptor Iα Enhances Allergen Specific Lymphocyte Responses in Experimentally Sensitized Dogs

Sho YASUNAGA[1)], Toshihiro TSUKUI[2)], Kenichi MASUDA[1)], Koichi OHNO[1)] and Hajime TSUJIMOTO[1)]

[1)]Department of Veterinary Internal Medicine, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Yayoi 1–1–1, Bunkyo-ku, Tokyo 113–8657 and [2)]Nippon Zenyaku Kogyo Co., Ltd., 1–1, Tairanoue, Sasagawa, Asaka-machi, Koriyama, Fukushima 963–0196, Japan

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ABSTRACT. Vaccination with a recombinant antigen fused to a targeting molecule is a potential strategy for inducing efficient immune responses. For the therapeutic purpose of allergic diseases in dogs, a DNA construct which expresses recombinant fusion protein with two functional domains, cytotoxic T lymphocyte antigen (CTLA-4) and Fcε receptor Iα, was developed to bridge antigen-presenting cells and IgE-allergen complex. The recombinant fusion protein expressed by the DNA construct was demonstrated to retain the ability to bind monocytes in PBMC and dog IgE, respectively. Additionally, the recombinant protein induced enhancement of allergen-induced lymphoproliferation in experimentally sensitized dogs under conditions of suboptimal allergen stimulation. These results indicated that the DNA construct could enhance allergen-induced immune responses *in vivo*, implying its usefulness for perspective application in immunotherapy in dogs.

KEY WORDS: allergic disease, canine, co-stimulatory molecule, DNA immunization, immunotherapy.

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Allergic diseases including atopic dermatitis and allergic bronchitis are caused by type 1 and type 4 hypersensitivities, and are one of the most frequently encountered diseases in veterinary medicine. As in humans, allergic diseases in dogs are caused by various allergens, and increasing numbers of dogs are suffering from dermal or respiratory symptoms derived from the IgE-mediated pathogenesis of disease. Specific immunotherapy has been applied to treat dogs with allergic diseases similar to homologous diseases in human medicine [22].

Allergen-specific immunotherapy, the administration of increasingly higher doses of allergen extract, is the only curative approach toward IgE-mediated allergies [17], and can induce the alleviation or resolution of sensitivity to an allergen. Although the complex mechanisms of immunotherapy are not well understood, proposed mechanisms include the induction of "blocking antibodies" [18], a reduction in the number of eosinophils in lesions and the release of proinflammatory mediators [5], the induction of suppressor cells [15], and the altering of the Th1-Th2 cytokine balance toward Th1 dominance [5, 10]. In spite of its curative potential, however, the application of immunotherapy to clinical cases of allergy is limited because of major drawbacks with the current protocol, some of which are more problematic in dogs than humans. The long duration necessary for therapy (months to years) and uncertain efficacy, which vary among reports as well as individuals, usually lead to poor compliance by patients (or owners in veterinary cases). Anaphylactic side effects are less problematic in dogs, but were reported to be a reason for cessation of therapy in some cases. It is considered that poor immunogenicity of the allergen, insufficient for the induction of Th1 dominance [5], and allergic responses against causative or other potential allergens included in the extract used for immunotherapy [17], are responsible for these drawbacks.

To improve the effectiveness and usability of current immunotherapy, a novel targeted DNA construct was developed for therapeutic purposes in the present study. The construct expresses the extracellular domain of cytotoxic T lymphocyte antigen-4 (CTLA-4) genetically fused to the extracellular domain of Fcε receptor Iα (FcεRIα), the mast cell-derived receptor capable of binding IgE [14]. CTLA-4 is a glycoprotein expressed on activated T cells that has strong ability to bind both CD80 and CD86, which are primarily expressed on antigen-presenting cells (APC) [3]. CTLA4-linked antigen is reported to induce strong immune responses by targeting APC through interactions with CD80 and CD86 [2]. Based on this background, the DNA construct was designed to bridge APC and IgE-allergen complex, inducing both anti-allergen responses and the anti-IgE idiotype. In the present study, the ability to bind dog IgE and CD80/CD86 of the recombinant fusion protein expressed by the DNA construct was investigated *in vitro*, and the influence of the recombinant on allergen-induced lymphoproliferation in experimentally sensitized dogs was evaluated. The preliminary findings indicate the possible effectiveness of the DNA construct for therapeutic applications as a novel targeted vaccin in dogs with allergic diseases.

MATERIALS AND METHODS

Plasmid construction: The extracellular region of canine CTLA-4 complementary DNA (cDNA) was generated by

* CORRESPONDENCE TO: OHNO, K., Department of Veterinary Internal Medicine, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1–1–1 Yayoi, Bunkyo-ku, Tokyo 113–8657, Japan.

RT-PCR amplification from total RNA of peripheral blood mononuclear cells (PBMC) of healthy dogs. The following PCR primers were designed to amplify nucleotides 23–510 of the reported sequence of canine CTLA-4 (Genbank accession number; AF143204): CTLABamF (GCGG-GATCCAGCCATGGCTGGCTTTG- GATT, adding a *Bam* HI site), CTLAXhoR (GCGCTCGAGTCAGTCA-GAATCTGGGCAA- GG, adding a *Xho* I site). As shown in Fig. 1A, the amplified fragment of about 500 bp was digested with *Bam* HI and *Xho* I, and ligated into pcDNA3.1/Myc-His(+) (Promega, Madison, WI) containing cDNA encoding mouse IgG2a Fc (Genbank accession number; J00479, nucleotides 292–1000) to promote dimerization for enhanced binding to CD80/CD86 [8] in the matching open reading frame (ORF). The cDNA encoding CTLA-4 joined to mouse IgG Fc (CTLA4-Ig) was further amplified by PCR using CTLAKpF (CGGGGTACCAGC-CATGGCTGGCTTTGGATT, adding a *Kpn* I site instead of *Bam* HI) and mIgNotR (ATAGTTTAGCGGCCGC-CCCGGAGTCC- GGGA, adding a *Not* I site), and the amplified fragment was ligated into the multicloning site of the eukaryotic expression vector pCAGGS [12] (pCAG-CTLA4-Ig).

The extracellular region of canine FcεRIα was amplified from cDNA of canine PBMC. The nucleotides 86–602 of the canine FcεRIα sequence (Genbank number; D16413) was amplified by PCR using the primers FcRNotF (ATTTGCGGCCGCTTGATACCT- TGAAACCTA, adding a *Not* I site) and FcRBglR (GGAAGATCTT-TACTCTTTTTTCACA- ATAAT, adding a *Bgl* I site). The amplified fragment of about 500 bp was digested with *Not* I and *Bgl* I, then ligated into pCAG-CTLA4-Ig downstream of mouse-IgG in a matching ORF (pCAG-CTLA4-Ig-FcεRIα; Fig. 1A).

Both the sequences and ORF of pCAG-CTLA4-Ig and pCAG-CTLA4-Ig-FcεRIα were confirmed using a conventional sequence analyzer (data not shown).

Transfection: Monolayers of COS-1 cells cultured in 6-

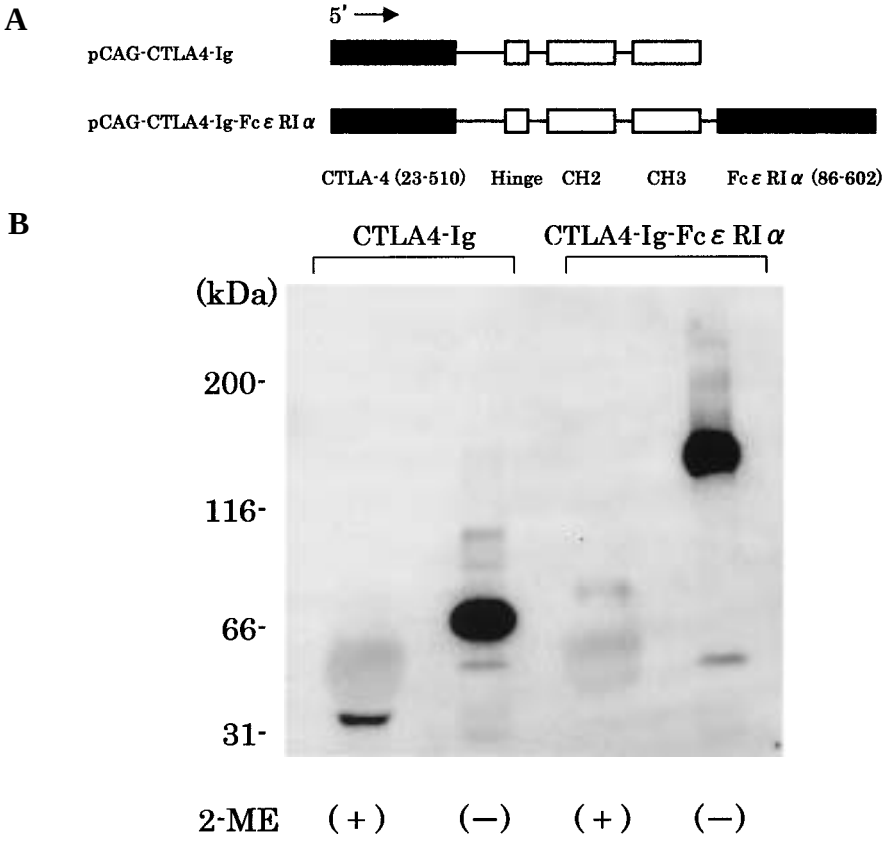

Fig. 1. (A) Schema of DNA constructs. All constructs were made on a pCAGGS backbone. Each rectangle represents an encoding region, and numbers in parentheses represent nucleotides in reported sequences of each counterpart. Empty rectangles (Hinge, CH2 and CH3) represent encoding regions derived from mouse IgG2a Fc to promote dimerization of produced recombinant. (B) Western blot analysis of expressed recombinant proteins. Culture supernatants of COS-1 transfectants, representative samples at 48 hr post-transfection, were run under reducing or non reducing condition. Recombinant chimeric proteins (CTLA4-Ig, CTLA4-Ig-FcεRIα) were detected using HRP-conjugated goat anti-mouse IgG Fc and chemiluminescence.

well cell culture cluster (COSTAR, Corning Incorporated, Corning, NY) were transfected with 2 μg of pCAG-CTLA4-Ig, pCAG-CTLA4-Ig-FcεRIα or parental pCAGGS as a negative control using Lipofectamine 2000 (Invitrogen, San Diego, CA) according to the manufacturer's instructions. Transfectants were cultured with Dulbbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/m*l* of penicillin and 100 μg/m*l* of streptomycin. Culture supernatants were collected at 48 and 72 hr post-transfection and tested by Western blot analysis.

Western blot and dot blot analysis: For the analysis of expressed recombinant protein, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis were performed as follows. Transfected COS-1 or cultured supernatants were mixed with Laemini Sample Buffer (Bio-Rad Laboratories, Hercules, CA) and 2% 2-mercaptoethanol (2-ME) for reduced conditions where indicated, boiled for 2 min, then loaded at 10 μl onto 5% SDS-polyacrylamide gel. Following SDS-PAGE, samples were transferred onto PVDF membrane (Hybond-P, Amersham Biosciences K.K.) and blocked with 5% skim-milk in phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBST) at 37°C for 60 min. After two washes with PBST, blots were probed with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Fc specific; Sigma-Aldrich, Saint Louis, MO), diluted in 3% skim-milk in PBS at 1:10,000 (at RT, for 30 min). After a wash with PBST, blots were developed with ECL Plus Reagents (Amersham Biosciences K.K.) and Hyperfilm ECL (Amersham Biosciences K.K.), according to the manufacturer's instructions. The recombinant proteins expressed by pCAG-CTLA4-Ig and pCAG-CTLA4-Ig-FcεRIα-transfected COS-1 cells were designated CTLA4-Ig and CTLA4-Ig-FcεRIα, respectively.

To examine whether the FcεRIα part of the recombinant protein could bind to dog IgE, dot blot analysis was performed. Ten micro-litters of hybridoma-derived dog IgE (BETHYL Laboratories, Montgomery, TX) diluted in 3 % skim-milk in PBST as 5 μg/m*l* was absorbed on a nitrocellulose membrane (Hybond ECL, Amersham Biosciences K.K.) and blocked with 5% skim-milk in PBST at 37°C for 30 min. The membrane was washed twice with PBST, then incubated with culture supernatants of transfected COS-1 cells diluted in 3% skim-milk in PBS at 1:20 (at RT, for 120 min). To verify the specificity of binding, supernatant containing CTLA4-Ig-FcεRIα was pre-incubated with 0.5 μg of hybridoma-derived dog IgE (4°C for 30 min) where indicated, then incubated with membrane. After two washes with PBST, the binding of recombinant protein with dog IgE was detected with HRP-conjugated goat anti-mouse IgG Fc (Sigma-Aldrich). For a positive control, IgE bound to membrane was detected with goat anti-dog IgE (ε chain specific; BETHYL Laboratories). Finally, membranes were developed with ECL Plus Reagents (Amersham Biosciences K.K.) and Hyperfilm ECL (Amersham Biosciences K.K.) as described above.

Experimental sensitization of dogs: Three intact beagles, aged 4 to 5 months, were sensitized to Japanese cedar (*Cryptomeria japonica*, *C. japonica*) pollen antigen (Cj) [20] as described previously [19]. Briefly, dogs were injected 100 μg of Cj conjugated with 20 mg of alum subcutaneously for each administration. Sensitization was performed twice at two-week intervals, and whole bloods samples were obtained 2 weeks after the last sensitization.

Peripheral blood mononuclear cells (PBMC): Heparinized whole blood samples were obtained from healthy or 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 × g for 40 min at RT, a layer of PBMC was collected and washed twice with PBS. Isolated PBMC were subjected to FACS analysis or a lymphocyte stimulation test.

Flow cytometric analysis: PBMC (4×10^6 cells) isolated from healthy dogs were resuspended in 100 μl of culture supernatant including CTLA4-Ig or CTLA4-Ig-FcεRIα, and incubated at 4°C for 30 min. Cells were washed twice with ice-cold PBS and further incubated with fluorescein isothiocyanate (FITC)-conjugated goat antimouse IgG (Fc specific; Sigma-Aldrich) diluted in PBS at 1:50 (4°C, for 30 min). Populations of monocytes, granulocytes and lymphocytes were fractionated according to forward scattering (FSC) and side scattering (SSC), and bindings of recombinant proteins in these populations were analyzed using FACSCaliber (BD Biosciences, San Jose, CA).

Lymphocyte stimulation test: To examine the effects of recombinant proteins on allergen-induced lymphoproliferation, a lymphocyte stimulation test (LST) was performed according to a previous report [11] with minor modification. Briefly, PBMC isolated from experimentally sensitized dogs were resuspended in DMEM supplemented with 5 % dog serum, 100 U/m*l* of penicillin and 100 μg/m*l* of streptomycin at a concentration of 1.25×10^6/m*l*, and 200 μl of cell suspension was assigned to each well of a 96-well microtiter plate. Cells were then stimulated with Cj (5 or 0.1 μg/m*l*) and/or 10 μl of culture supernatant containing CTLA4-Ig or CTLA4-Ig-FcεRIα, and incubated at 37°C for 72 hr. The volume of culture supernatants was determined according to the result of flow cytometric analysis so that sufficient monocytes in PBMC were bound with CTLA4-Ig-FcεRIα. After incubation with 1 μCi/well of ^{3}H-thymidine for the final 18 hr, the incorporated radioactivity (cpm) was measured with a liquid scintillation counter. All samples were subjected to triplicate examinations, and reactivity to Cj and/or culture supernatant was expressed as a Stimulation index (SI), the ratio of the mean cpm of the stimulated culture to that of unstimulated culture. A SI of more than 2.0 was considered significant.

RESULTS

Expression of CTLA4-Ig constructs in mammalian cells: The expression of pCAG-CTLA4-Ig and pCAG-CTLA4-Ig-FcεRIα in eukaryotic cells was examined by transfection of

COS-1 cells. Western blot analysis using anti-mouse IgG Fc confirmed the expression of CTLA4-Ig and CTLA4-Ig-FcεRIα in both cell lysate (data not shown) and culture supernatant (Fig. 1B) 48 and 72 hr post-transfection, whereas the transfectant with parental pCAGGS showed no protein production (data not shown). As shown in Fig. 1B, the expressed recombinant proteins, CTLA4-Ig and CTLA4-Ig-FcεRIα, were about 40 kDa and 90 kDa under reducing conditions, as expected from the length of the encoding sequences, although expression of CTLA4-Ig-FcεRIα was quite weak probably due to recognition of secondary epitopes by polyclonal antibody used for detection. Under non-reducing conditions, the recombinant proteins were about 80 kDa and 180 kDa, respectively, indicating both products were expressed in dimerized form as expected.

Ability of the recombinant fusion proteins to bind dog IgE and monocytes: The binding ability of the CTLA-4 part of both recombinant proteins CTLA4-Ig and CTLA4-Ig-FcεRIα was examined by flow cytometric analysis. PBMC isolated from healthy dogs were used as a source of dog CD80 and CD86, and incubated with culture supernatants of COS-1 transfectants. As shown in Fig. 2A and 2B, both CTLA4-Ig and CTLA4-Ig-FcεRIα bound monocytes in PBMC, although anti-mouse IgG Fc alone showed a certain baseline suggesting possible binding to Fc receptors on monocytes. No binding of either recombinant protein was detected among other cell populations in PBMC (data not shown).

Binding of the FcεRIα moiety with dog IgE was examined by dot blot analysis. As shown in Fig. 3, the binding of CTLA4-Ig-FcεRIα to membrane-bound dog IgE was detected by dot blot analysis, whereas that of CTLA4-Ig was not. The positive spot on CTLA4-Ig-FcεRIα disappeared when supernatant was pre-incubated with dog IgE. The binding specificity of CTLA4-Ig-FcεRIα indicated that the process was mediated through interaction of the FcεRIα

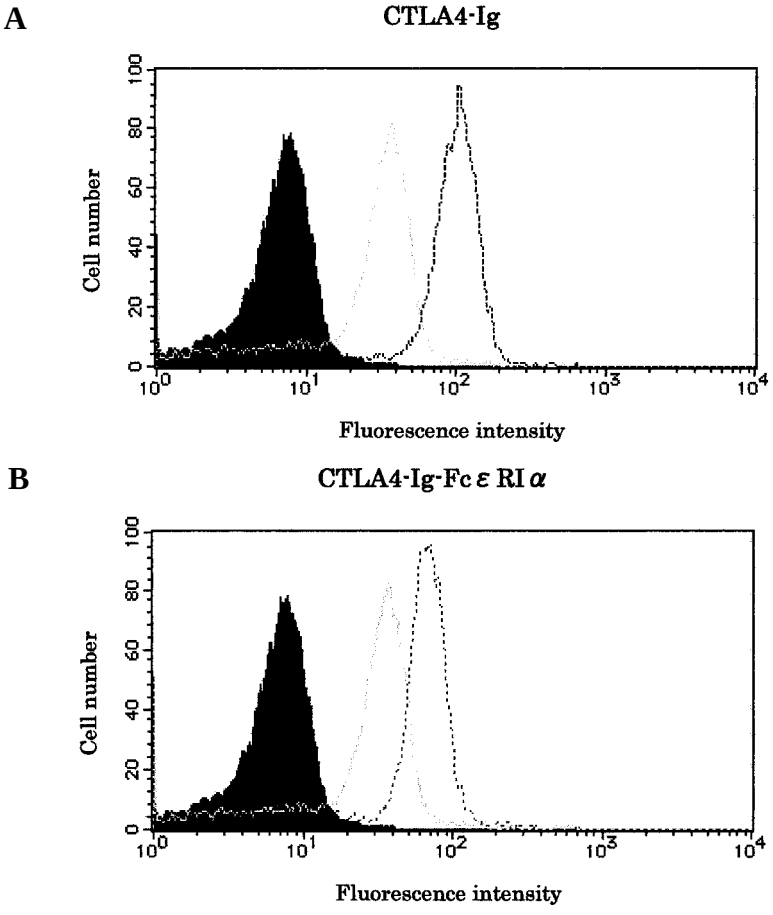

Fig. 2. Flow cytometric analysis for detection of the binding of CTLA-4 counterparts. PBMC of healthy dogs were incubated with culture supernatant of transfectants containing CTLA4-Ig (A) or CTLA4-Ig-FcεRIα (B), followed by incubation with FITC-conjugated goat anti-mouse IgG Fc. The histograms depict the logalithmatic fluorescence intensity of the gated monocytes, stained with recombinant proteins and anti-mouse IgG (dotted line), anti-mouse IgG only (thin line), and without antibody (filled area).

CTLA4-Ig	−	−	+	−	−
CTLA4-Ig-Fc ε RI α	−	−	−	+	+
Anti-mouse IgG	−	+	+	+	+
Anti-dog IgE	+	−	−	−	−
Pre-incubation with dog IgE	−	−	−	−	+

Fig. 3. Dot blot analysis indicating the binding of FcεRIα counterpart and dog IgE. Membrane-bound dog IgE was incubated with CTLA4-Ig or CTLA4-Ig-FcεRIα followed by HRP-conjugated goat anti-mouse IgG Fc and chemiluminescence. On the blot at the right end, CTLA4-Ig-FcεRIα was pre-incubated with dog IgE to inhibit specific binding between CTLA4-Ig-Fc εRIα and membrane-bound dog IgE. HRP-conjugated goat anti-dog IgE and chemiluminescence were used as a positive control.

moiety with dog IgE.

Effect of CTLA4-Ig-FcεRIα on lymphoproliferation of PBMC of Cj-sensitized dogs: Since the former experiments indicated that two functional components of CTLA4-Ig-FcεRIα retain the ability to bind the respective ligands, it is considered that the recombinant fusion protein is capable of bridging APC and allergen-IgE complex, inducing a enhanced allergen-induced lymphoproliferation. To evaluate the biological activity of CTLA4-Ig-FcεRIα, modified LST was performed by adding the recombinant proteins into cultures of PBMC from experimentally Cj-sensitized dogs as models of allergic diseases. On stimulation with 5 μg/m*l* of Cj, the regular concentration used for LST in our protocol, all stimulated cultures showed strong blastogenic responses (SI ≥ 2.0), and no effect of recombinant proteins was found (Fig. 4A). On the other hand, under stimulation with 0.1 μg/m*l* of Cj, a sub-optimal concentration for induction of blastogenic responses, strong responses were still detected in cultures with CTLA4-Ig-FcεRIα of two dogs (Fig. 4B). In all dogs, addition of CTLA4-Ig or CTLA4-Ig-FcεRIα alone in culture did not induce any blastogenic response (data not shown).

DISCUSSION

The efficient induction of immune responses by DNA vaccines which encode "targeted" antigen has been reported in a variety of disease models including tumors [9] and infectious diseases [4], but is rarely reported in allergic diseases. In these studies, various molecules including cytokines (e. g., IL-2, IL-4), granulocyte-macrophage colony-stimulating factor (GM-CSF) and L-selectin were used as a carrier to target antigens. CTLA-4 is one of the most potent candidates for targeting molecules. The mechanisms of action of CTLA4-targeted vaccines have not been well explained, but may include an enhanced intake of linked-antigen by APC through interaction with specific ligands (CD80 and CD86), thereby enhancing the processing and presentation of antigen [2].

Based on this background, a novel DNA construct, pCAG-CTLA4-Ig-FcεRIα, which utilizes CTLA4-CD80/CD86 interactions, was developed in the present study for therapeutic application to allergic diseases. The extracellular domain of FcεRIα, a high affinity receptor for IgE, was fused to CTLA-4 to capture IgE and allergen bound to it. The potential uptake of allergen and IgE through CTLA4-Ig-FcεRIα would induce not only the enhancement of allergen-specific immune responses which promote efficient immunization in immunotherapy, but also the production of the anti-IgE idiotype which would be beneficial for suppression of pathogenic IgE.

In the present study, we examined CTLA4-Ig-FcεRIα, recombinant protein expressed by pCAG-CTLA4-Ig-Fcε RIα, in terms of its binding ability and biological activity. CTLA4-Ig-FcεRIα could bind to monocytes in PBMC and dog IgE respectively, suggesting that it would bridge APC and IgE as expected. Although the specificity of CTLA-4 to bind monocytes had not been verified, the limited binding of

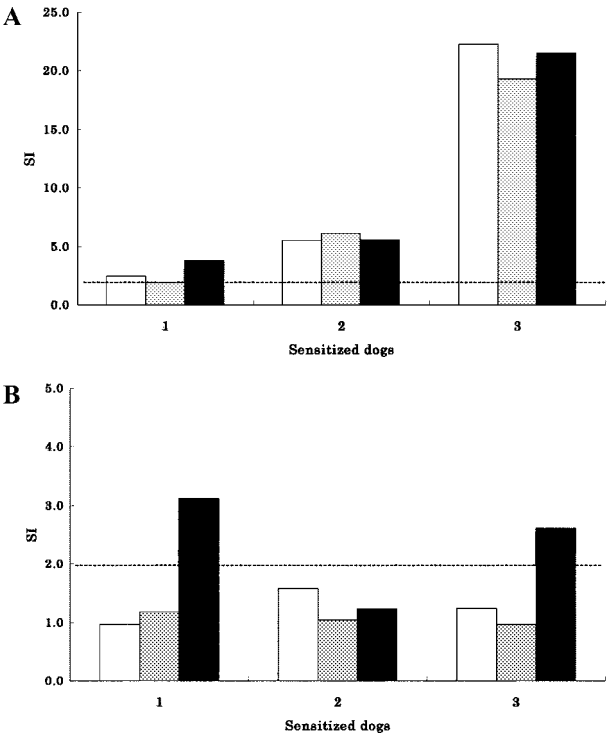

Fig. 4. Cj-induced blastogenic responses of PBMC from sensitized dogs and biological effects of recombinant proteins. PBMC of sensitized dogs were stimulated with 5 μg/m*l* (A) or 0.1 μg/m*l* (B) of Cj, together with recombinant proteins; CTLA4-Ig (gray bar), or CTLA4-Ig-FcεRIα (filled bar) in culture. As a control, culture medium without recombinant protein was used (empty bar). Proliferations of cells were expressed as SI, the ratio to unstimulated culture. Mean results of three experiments were shown. The threshold of blastogenesis (SI=2.0) is shown as a dotted line.

CTLA4-Ig-FcεRIα (and CTLA4-Ig) to monocytes in PBMC suggests the process was mediated by CTLA4-CD80/CD86 interactions as CD80 and CD86 are dominant on the monocytes of healthy individuals [1, 6]. In spite of the stable expression of CD80 and CD86 on B cells [1, 6], the binding of recombinant proteins on lymphocytes could not be detected in this study, possibly due to the small number of B cells in PBMC or low level of expression of these molecules.

In the results of modified LST, enhancement of the allergen-induced proliferation of lymphocytes by CTLA4-Ig-FcεRIα was observed at a sub-optimal allergen concentration (0.1 μg/m*l*), suggesting the CTLA4-Ig-FcεRIα could enhance allergen uptake by APC by utilizing autologous IgE produced by B cells in PBMC. The production of IgE in supernatants was not examined in the present study, but is thought to be low in our 3 day cultures. However, a previous study on CTLA4-targeted vaccine reported that very low concentration of recombinant CTLA-4 could induce remarkable responses to antigen [9], therefore it was considered that extremely low concentrations of IgE would be sufficient to enhance allergen uptake. No enhancement was found at a regular allergen concentration (5 μg/m*l*), indicating that any enhancement of lymphoproliferation by CTLA4-Ig-FcεRIα would have been masked under sufficient allergen-stimulation. For prospective application in immunotherapy, further investigation would be necessary to evaluate the CTLA4-specificity of the binding of recombinant protein, and effects on antigen-induced immune-responses *in vivo*, as well as the enhancement of antigen-induced immune responses through non-covalent links such as FcεRIα-IgE, the first reported among targeted vaccines.

In conclusion, we developed a novel DNA construct, which expresses recombinant fusion protein of CTLA-4 and FcεRIα to bridge APC and IgE-allergen complex. Preliminary *in vitro* studies confirmed that this recombinant protein could bind both monocytes in PBMC and dog IgE, and enhanced allergen-induced lymphoproliferation in experimentally sensitized dogs. Previous reports including our study in dogs described enhanced expression of CD80 and CD86 in immune cells in allergic patients [13, 16, 21], suggesting the potential effectiveness of CTLA4-targeted vaccines for application in immunotherapy. Further investigation of this novel strategy will hopefully provide options for the treatment of allergic diseases.

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