

PRODUCTION OF A RECOMBINANT CANINE IgE MOLECULE

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ABSTRACT

Immunoglobulin E (IgE) is a key molecule in immediate allergic reactions in dogs, and it is used in vitro for detection of allergen-specific IgE employing anti-dog IgE antibodies. Usually, these reagents are developed against IgE purified from sera that frequently are contaminated with other immunoglobulins, cross-reaction with which could produce false positives. Molecular biology techniques now allow the production of immunoglobulins free of contamination with other isotypes. The aim of this study was to produce recombinant dog IgE (rIgE) using the known sequence of its gene that comprises the domains CH2-CH3-CH4 of the constant region of heavy chain. A mammalian cell system was employed in order to maintain the glycosylation motifs of the molecule. rIgE was affinity purified and characterized in order to check its molecular weight and structure in SDS-PAGE and immunoblotting assays. The rIgE represented a minority protein in the supernatant of transfected cells. The purification needed several washing steps, and the elution required high concentration of imidazole (500 mM). rIgE was specifically recognized by an anti-dog IgE-peroxidase polyclonal antibody, showing an apparent molecular weight of 50 kDa in the presence of beta-2-mercaptoethanol and a molecular weight of 100 kDa in non-reducing conditions consistent with the predicted molecular for mono and dimeric forms. The company Alergovet has produced a rIgE that maintains native IgE epitopes. This rIgE could be used to produce new monoclonal antibodies against the Fc region of dog IgE that would be free of contamination and cross-reaction with other immunoglobulins.

GENEBANK Acc. Number AAAS6797 [Patel M. et al. Immunogenetics 41 (5), 282-286 (1995)]

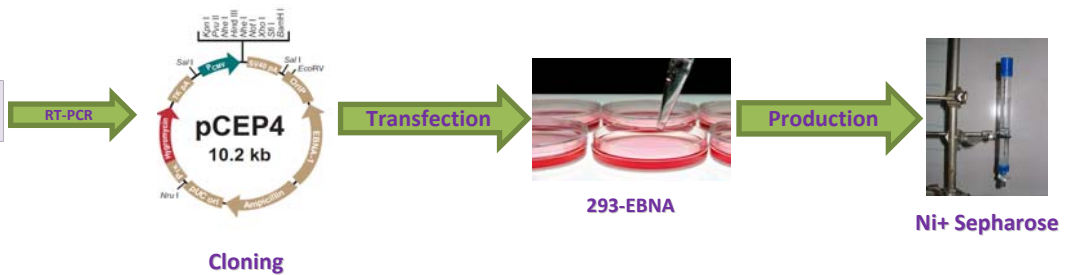


Figure 1. Cloning of CH2-CH3-CH4 region of heavy chain of dog IgE. Total mRNA was extracted and purified from peripheral blood mononuclear cells (PBMC) obtained from atopic dogs with specific IgE high levels (dust mites and grasses). One µg of total mRNA was reverse transcribed using a random hexamer mix as primer. sscDNA was used as template in PCR in order to amplify the entire encoding CH2-CH3-CH4 region of heavy chain of dog IgE (Patel et al., 1995). The 5' primer contained an 18-hexanucleotide region encoding a histidine hexapeptide. The PCR products were purified and cloned into the expression vector pCEP4 (Invitrogen). The hygromycin resistance gene was replaced with the puromycin resistance gene for cell selection purposes.

The selected clone was sequenced to check integrity. Human embryonic kidney cells (293-EBNA) were transfected with the plasmid using Lipofectamine 2000 (Invitrogen) following manufacturer recommendations. Growing cells were expanded and conditioned supernatant were collected every 5 days. Cellular debris was removed by centrifugation before purification events. The production of recombinant IgE (rIgE) was checked by ELISA and western-blot. rIgE was purified by affinity using Ni+ Sepharose (General Electric) as matrix and Imidazole as eluent, according to the manufacturer's instruction. rIgE-containing fractions were pooled and dialyzed against PBS 0.01 M pH 7.2 using a membrane with a cut-off of 12500 daltons (Sigma).

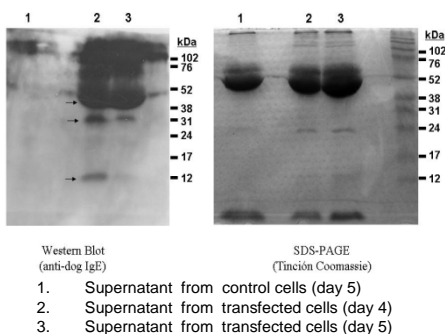
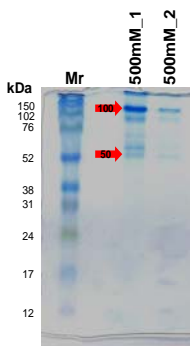
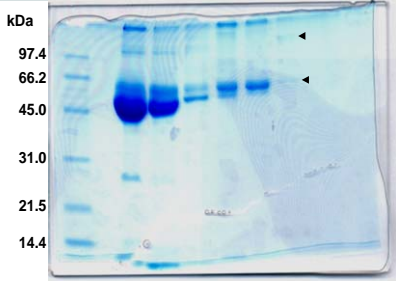


Figure 2. Detection of rIgE in supernatants of transfected cells. Supernatants from cellular cultures of 293-EBNA cells transfected with dog-rIgE plasmid were obtained 4 or 5 days after transfection. Other cultures were transfected with plasmid pCEP4 without IgE sequence and its supernatants recovered the 5th day. The three samples were assayed by non-reducing 15% SDS-PAGE and, in parallel, by western-blot with an specific anti-canine IgE HRP labeled antibody. The protein profiles of three tested supernatants are similar in SDS-PAGE. However, anti-canine IgE antibody only produced signal in those supernatants of dog-rIgE plasmid transfected cells (wells 2 and 3). Antibody recognized 4 different bands of around 12, 26, 50 and 100 kDa.

rlgE Purification

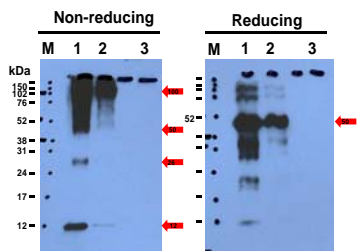
Non-binding
20 mM (1st)
20 mM (2nd)
100 mM (1st)
100 mM (2nd)
500 mM (1st)
500 mM (2nd)

Anti-dog IgE ELISA: 0.05 0.01 0.01 0.35 0.97 2.32 1.69



A. 12% SDS-PAGE (non-reducing)

western-blot anti-dog IgE



M. Markers

1. Eluted 500 mM₁
2. Eluted 500 mM₂

B. 3. Control: non-transfected cells supernatant

Figure 3. Purification of canine rlgE by affinity. Cell culture supernatants were centrifuged at 1,200 rpm during 10 min in order to remove cells and cellular debris. One milliliter of Ni⁺ Sepharose beads (General Electric) in PBS 0.1 M was added per 50 ml of supernatant and incubated overnight at 4°C on a shaker. The recombinant protein was eluted with increasing concentrations of Imidazole (General Electric) in 6 elution steps: 20 mM (x2), 100 mM (x2) and 500 mM (x2). The figure shows the protein profiles (non-denaturing 12% SDS-PAGE) and the relative amount of rlgE assayed by ELISA (results in optic density arbitrary units) of each fraction. The elution of rlgE was reached with Imidazole at 100 mM (O.D. = 0.35) but the highest amount of protein was reached with 500 mM (O.D. = 2.32). The 500 mM fractions showed the highest amount of recombinant IgE with the less complex protein profile

Figure 4. Structure of purified recombinant canine IgE. The 500 mM₁ and 500 mM₂ fractions were pooled and dialysed against PBS 0.1M pH 7.2 using a membrane with a cut-off of 12,500 dalton (Sigma). Finally, the pools were freeze-dried in order to reduce the volume. Pools were studied by SDS-PAGE in non-reducing conditions (A.) and by western-blot in reducing and non-reducing conditions using an specific anti-dog IgE antibody HRP conjugated (B.).

All protein bands contained in both pools (500 mM₁ and 500 mM₂) were detected by the specific dog-IgE antibody as shows figure 4.B. However, antibody didn't recognize proteins in the assayed control supernatant. Then, both pools contain purified recombinant canine IgE without contamination from other supernatant's proteins. Nevertheless, at least four different bands are observed in 500 mM₁ pool that show MWs of 12, 26, 50 and 100 kDa. This complex protein profile observed in both pools could be explained if those different bands correspond to different structural conformations of the same recombinant IgE molecule. In case the deduced molecular weight (MW) of the polypeptide core is 50 kDa (according to its aminoacidic sequence) the 26 and 12 kDa bands could be recombinant IgE degraded during cell culture and purification process. Moreover, the proteins above 50 kDa (100 kDa) could be explained as inter-chain disulfide bridges establishment between two rlgE monomers of 50 kDa. This hypothesis was confirmed by results of western-blot in reducing conditions (treatment with beta-2-mercaptoethanol) where an increase of 50 kDa band and decrease of 100 kDa band staining were observed.

CONCLUSIONS:

- ✓ ALERGOVET has produced a purified rlgE that maintains native IgE epitopes.
- ✓ rlgE is being used as antigen in the development of new monoclonal antibodies without IgG interferences.

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