

# The effect of pea albumins on immune response in mice

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## Abstract

The aim of presented studies was to determine the effect of total pea albumins on mice immune system response. Oral and intraperitoneal (i.p.) route of antigen administration were checked and three different adjuvants (Freund adjuvant, alum and cholera toxin) were used. All three groups showed enhancement of serum IgG and IgA antibodies production. Using cholera toxin (CT) to oral immunization with pea albumins gave comparable antibodies level to intraperitoneal immunizations, and the best T cell response.

Group orally immunized with pea albumins (PA) with CT as adjuvant resulted in high percentage of CD4 T cells population. We concluded, that the oral model of Balb/c mice immunization with antigen and CT as adjuvant, could be used to experiments determining food proteins immunogenicity and response of effector tissue of mucosal immune system.

**Key words:** pea albumins, immunization, adjuvants, antibodies, CD4, CD8.

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## Introduction

Administration of foreign proteins – potential allergens usually affect antibodies class G and A production. When allergy is induced antibodies IgE have to be produced and inflammatory reactions are beginning [1, 2]. It is proven that the dose of allergen, as well as adjuvant used and route of administration (oral or intraperitoneal) significantly affect specific antibodies level [3]. It is also known that changing the route of immunization and dose of given allergen can shift immune response from tolerance to immunity [3]. Knowledge on those mechanisms is very important for preventing and treating food allergy. Presented paper shows difference in mice immune system response due to varied pea albumin administration. Pea albumins have been selected as they are good source of valuable nutritional compounds [4] and they are present in traditional home cooking in number of countries, for instant Poland or UK. In the times, while meat substitute was looked for, the pea was found to be the one after soya due to high nutritional value and high proteins content [5]. Pea albumins, especially fractions P1 and P2 with molecular masses of 6.5 kDa and 26 kDa [6, 7] are also considered as fractions with high allergenic potential. The aim of the presented studies was to deter-

mine the effect of total pea albumins on mice immune system response, to choose between three ways of immunization the best model for checking their immunogenicity.

## Material and methods

Pea (*Pisum sativum*) seeds of Ramrod cultivar were obtained from the Institute of Plant Farming in Łągiwniki. Seeds were milled in a WZ-1 grinder (ZBPP Sadkiewicz Instruments, Poland) and sieved through a standard 0.40 mm sieve (NAGEMA, Germany).

## Albumins extraction

Pea flour (50 g) was extracted with 1000 ml of H<sub>2</sub>O d.d. at 4°C for 24 h, then the extract was centrifuged (22 500 × g, 4°C, 15 min). Supernatant containing total albumins was dialyzed against distilled water for 48 h. Then, the dialysed product was lyophilized, and obtained total pea albumin powder was further referred to as the pea albumins [8].

## HPLC analysis

The purity and molecular masses (MM) of isolated proteins were checked in Shimadzu HPLC System. The pro-

teins were eluted from the Biosep SEC-S 2000 column (75 × 7.80 mm) with 25 mM phosphate buffer pH 7.0 at flow rate of 1 ml/min. The detection was performed with UV at 214 and 280 nm. Molecular weight marker kit for gel filtration chromatography (MM range: 6500–66 000) was used as the molecular mass standards.

### Mice experiments

Balb/c mice, 6- to 8-week old, were purchased from the Medical Research Center PAS, Warsaw. Mice were immunized with pea albumins (PA) in three ways: group I, intraperitoneally on day 0, 7, 14 with 100 µg of PA with 100 µl of Complete Freund's Adjuvant (FA) for first immunization and 100 µl of incomplete FA for next two immunizations; group II orally with 200 µg PA with 61.5 µl Alum (Sigma) by 10 consequence days and group III with 200 µg PA with 5 µl Cholera Toxin (CT) for first immunization and 2.5 µl CT for the next, for 10 consequence days. Oral doses were administered with a 21-gauge gavage needle attached to a 1 ml tuberculin syringe directly to the stomach. On 14, 21, 28 and 35 day from the first immunization, blood and fecal samples were collected for future IgG and IgA level analysis [9]. Each experimental group consists of 10 mice. The Local Care Use of Animal Committee (authorization 96/2010/N) approved animal handling and experimental procedures.

### Anti-PA antibodies level determination

To evaluate PA-specific endpoint Ab titers in serum and fecal extracts standard ELISA method was used. Following washing, horseradish peroxidase-conjugated secondary goat-anti-IgG and – IgA (Sigma) were added to the wells and incubated for 90 min at 37°C. After washing, ABTS substrate (Moss) was added and incubated for 60 min at room temperature. Endpoint titers were defined as the highest reciprocal of dilution of sample, giving an absorbance at OD415 above 0.100 OD units above negative control [9].

### Lymphocyte isolation

On day 35 mice were killed and tissue samples from spleen and mesenteric lymph nodes were collected for lymphocytes isolation [9, 10].

Cells at a concentration of  $2 \times 10^5$  cell per 100 µl were cultured in RPMI 1640 medium (Sigma) supplemented with 10% heat inactivated fetal bovine serum (FBS, GIBCO), 1% non-essential amino acids (Sigma) and 1% penicillin-streptomycin solution (Sigma), at 37°C in the atmosphere of 5% CO<sub>2</sub>. Lymphocytes were cultured for 4 days in presence of 1 mg/ml PA.

### Flow cytometry analysis

Spleen (SPL) and mesenteric lymph nodes (MLN) lymphocytes were stained with FITC-conjugated anti-mouse CD4 mAbs (BD Pharmingen, clone H123.19) versus phycoerythrin (PE)- conjugated anti-mouse CD8 mAb (BD Pharmingen, clone 53-6.7). Two-color Flow cytometry (FCM) analysis was performed on gated lymphocyte cells [11].

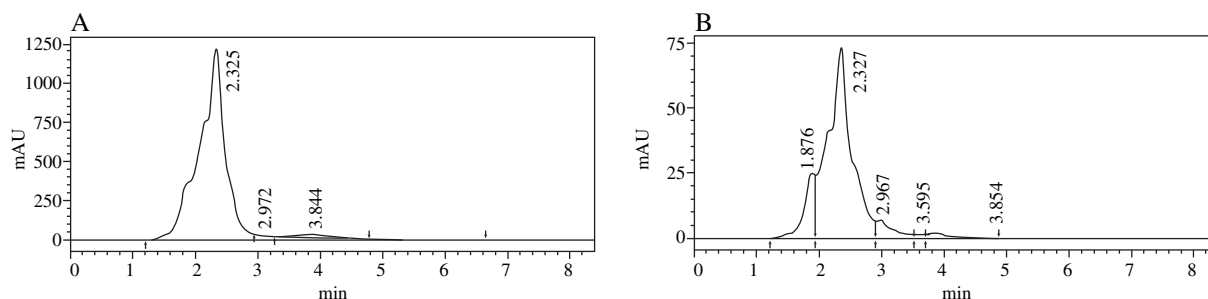
### Statistical analysis

One-way ANOVA followed by a Tukey test was applied to evaluate statistical differences between antibodies titres in experimental groups. The flow cytometry data were analyzed by one-way ANOVA followed by a Holm-Sidak method. Results were considered to be statistically significant if *p*-value was less than 0.05.

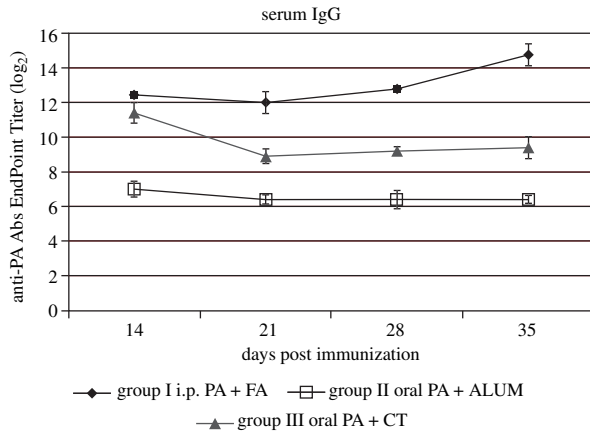
## Results

Pea albumins were extracted from the flour according to the method described above. On the HPLC chromatogram of pea albumins extract (Fig. 1) there was found one main peak eluted at 2.32 min of chromatographic separation and corresponding to the protein of molecular mass about 12 kDa and 3 minor peaks corresponding to the proteins of molecular masses lower than 12 kDa.

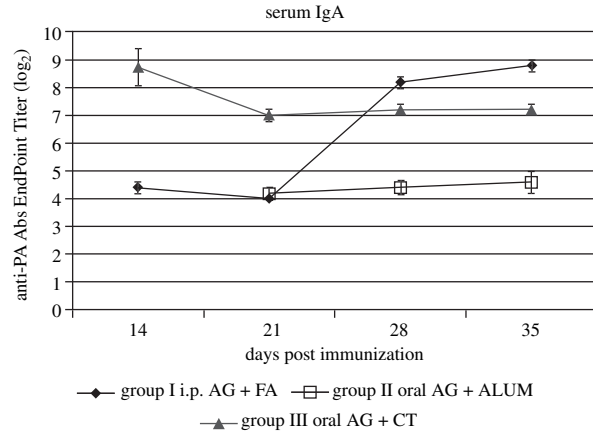
All experimental groups showed production of PA-specific immunoglobulins class G (Fig. 2). Specific IgG titer risen in group intraperitoneally immunized during the time of experiment from  $2^{12} \pm 0.2$  after 14 days to  $2^{15} \pm 0.6$  after 35 days. Oral PA administrations in the presence of CT as adjuvant induced specific IgG titer  $2^{11} \pm 0.6$ . During the next 7 days titre decreased to the level about 29 and was



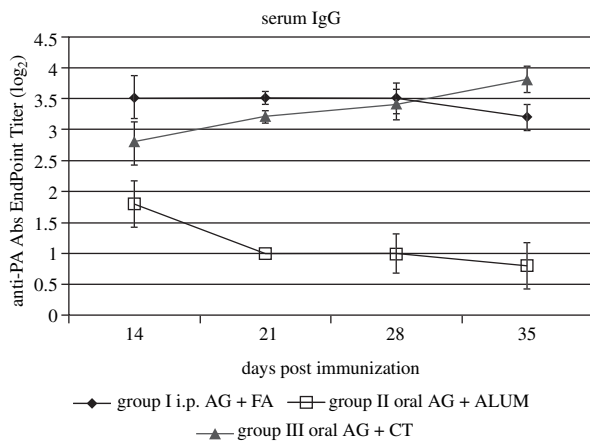
**Fig. 1.** HPLC analysis of pea albumins extracts (BiosepSEC-S 2000 column 75 × 7.80 mm): A – chromatogram at 214 nm, B – chromatogram at 280 nm



**Fig. 2.** Serum IgG Anti-PA antibodies EndPoint Titre after immunization with: GR I – i.p. 200 µg PA with FA, GR II – oral 100 µg PA with Alum, GR III – oral 100 µg PA with CT



**Fig. 3.** Serum IgA Anti-PA antibodies EndPoint Titre after immunization with: GR I – i.p. 200 µg PA with FA, GR II – oral 100 µg PA with Alum, GR III – oral 100 µg PA with CT



**Fig. 4.** Fecal IgG Anti-PA antibodies EndPoint Titre after immunization with: GR I – i.p. 200 µg PA with FA, GR II – oral 100 µg PA with Alum, GR III – oral 100 µg PA with CT

constant by the next 14 days. The lower induction of anti-PA IgG (Fig. 2) and IgA (Fig. 3) was found in group immunized orally in the presence of alum as adjuvant. Interesting differences were found in induction of specific IgA. Oral immunization with CT resulted in high titre  $28 \pm 0.7$  after 14 days of experiment and remained close to that value by the next 21 days (Fig. 3). Intraperitoneal immunizations induced specific IgA on detectable level on 21 day of experiment and then the increase up to  $28 \pm 0.2$  was observed by the next 14 days.

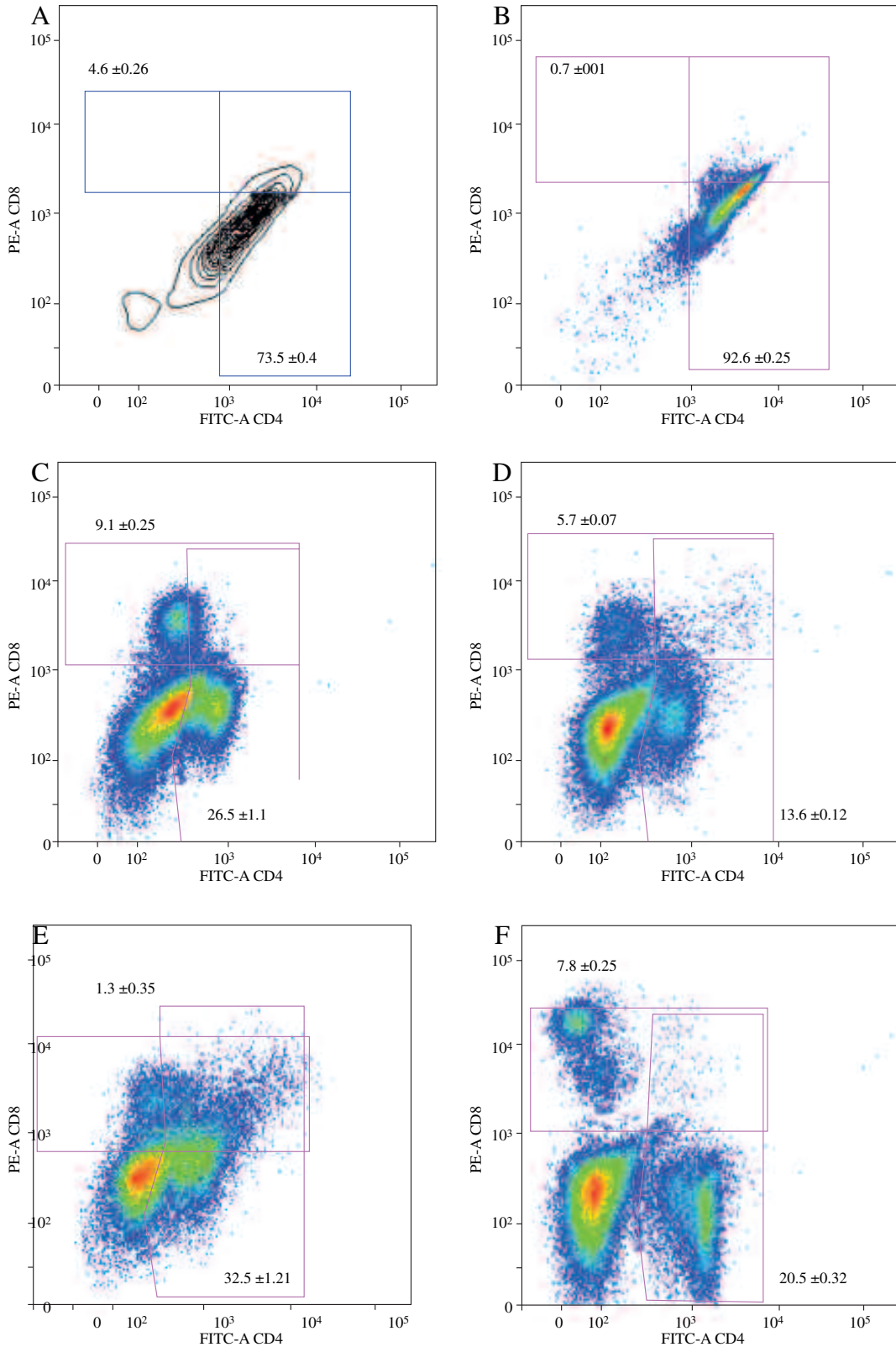
Fecal samples IgA level was low in all experimental groups (Fig. 4). Oral immunization with alum gave statistically significant differences in IgA level as compared to group I and III ( $p < 0.05$ ).

To check CD4 and CD8 T cells distribution after different immunizations, lymphocytes from spleen and mesen-

teric lymph nodes (MLNs) were isolated and stained (Fig. 5). MLNs second after Payer’s Patches are thought to be the main site for oral tolerance induction [12]. As results show CD4 and CD8 T cells percentage are strictly correlated with route of immunization and used adjuvant. Oral immunization gave the higher level of induced CD8 T cells in MLNs when alum was used as well as CT,  $9.1 \pm 0.25$  and  $13 \pm 0.35$ , respectively (Fig. 5C, E). Intraperitoneal immunization gave statistically different (for  $p \geq 0.001$ ) level of MLNs CD8,  $4.9 \pm 0.26\%$ . For MLNs CD4 statistically important differences (for  $p = 0.04$ ) were found between oral and i.p. immunized groups (Fig. 5A, C, D). There were no differences between using Alum or CT as adjuvant. Similar situations were found after phenotyping SPL lymphocytes (Fig. 5B, D, F). Using CT as adjuvant gave the best percentage of CD8,  $7.8 \pm 0.25$  compare to i.p. group  $0.7 \pm 0.01$  and group II  $5.7 \pm 0.07$  ( $p \geq 0.001$ ). Intraperitoneal immunization significantly increased CD4 induction in group III (Fig. 5A, B) as compared to group II and III where alum and CT were used (Fig. 5C–F) ( $p \geq 0.001$ ).

## Discussion

Presented experiments showed that each used route of immunization gave positive results for antibodies production. High level of serum specific IgG and IgA were found in all groups. While determining secretory IgA levels, it was found that using alum for oral immunization is not enough efficient. Alum is widely used adjuvant in human vaccines [13]. It has been found to be able to suppress Th2 cytokine production locally [14] and generate specific antibody and T cell response [15, 16]. In presented models, level of secretory IgA induced in group II (oral Ag+Alum) was the lowest. Using CT as adjuvant during oral immunization gave not significant differences as compared to i.p. immunization. Induction of CD4 T cells population is also the most



**Fig. 5.** CD4 and CD8 T cells distribution in MLN and SPL lymphocytes isolated from: A, B – GR I – i.p. 200 µg PA with FA; C, D – GR II – oral 100 µg PA with Alum; E, F – GR III – oral 100 µg PA with CT

efficient in group III (Fig. 5E, F), however i.p. immunization are invasive and do not correlate with natural occurring food antigen absorption. There are a number of examples using mucosal adjuvant CT in OVA experiments to induce tolerance [3, 17] or for inducing tetanus protection [18]. It is proven that CD4 T cells are precursors of subpopulation of regulatory cells, which are able to modulate immune response, inhibit immune diseases at sites of inflammation or infection [19, 20]. What is a very important, regulatory T cells have effect in the lymph nodes and in the target tissue [20], therefore it is very important to induce them during immunization.

Presented results have proven that using CT to oral immunization with pea albumins gave comparable antibodies level to intraperitoneal immunizations and the best T cell response.

## Conclusions

Oral model of Balb/C immunization with antigen and CT as adjuvant can be used to experiments determining food proteins immunogenicity and their effect on effector tissue response of mucosal immune system.

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