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ЭФФЕКТ β-АЛАНИНА НА ГУМОРАЛЬНЫЙ ИММУННЫЙ ОТВЕТ В НИЗКОДОЗОВОЙ МОДЕЛИ АЛЛЕРГИИ

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Резюме. В настоящее время усилия многих научных групп в мире направлены на поиск новых факторов, запускающих процесс аллергической сенсибилизации, связанный с синтезом IgE в ответ на безвредные аллергены. По современным данным, продукция тканевых цитокинов индуцируется в тканях через алармины, что, в свою очередь, вызывает проаллергический иммунный ответ. Ранее мы показали, что β-аланин может быть потенциальным алармином, способным стимулировать продукцию тканевых цитокинов. Целью настоящей работы было определение вклада β-аланина в гуморальный иммунный ответ на модели низкодозной аллергии. Мышей BALB/c иммунизировали в область холки рекомбинантным белком Asp f 2 или коммерческим овальбумином (OVA) три раза в неделю с добавкой β-аланина или без него. Чтобы уточнить эффект β-аланина, его сравнивали с действием α-L-аланина – изомера, который не является лигандом рецептора MrgD, а также β-аминоизобутирата, сходного по аффинности к MrgD с β-аланином. Согласно нашим данным, β-аланин стимулировал специфическую продукцию IgE и IgG1 при кратковременном курсе (7 иммунизаций) и повышенное сродство антител после долгосрочной иммунизации (14 инъекций) при использовании менее иммуногенного белка Asp f 2. В случае применения высокоиммунного белка OVA, вклад β-аланина был значимым только в плане аффинности антител. Таким образом, β-аланин ускоряет продукцию специфического IgE в случае применения белка с низкой иммуногенностью. Вклад β-аланина в продукцию специфического IgE не был связан с активацией специфического рецептора MrgD, поскольку β-аминоизобутират – другой лиганд этого рецептора – не оказывал сходного эффекта на гуморальный иммунный ответ. Воздействие β-аланина на продукцию IgG1 также представляется независимым от рецептора MrgD, поскольку α-L-аланин – известная протеиногенная аминокислота также повышала продукцию специфического IgG1. Эффект β-аланина на гуморальный иммунный ответ может быть связан с его неспецифическим действием, например, с его способностью вызывать окислительный стресс путем блокады тауринового транспортера, или со способностью стимулировать клеточный метаболизм.

Ключевые слова: бета-аланин, IgE, низкие дозы, аффинность антител, иммуногенность, MrgD-рецептор

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EFFECT OF β-ALANINE ON HUMORAL IMMUNE RESPONSE IN LOW-DOSE ALLERGY MODEL

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Abstract. At the present time, the efforts of many research groups around the world are aimed at finding new factors triggering the allergic sensitization process linked with IgE synthesis to harmless allergens. According to the recent data, production of tissue cytokines is induced in tissue cells by alarmins, thus, in turn, eliciting pro-allergic immune response. Previously we have shown that β-alanine could be a potential alarmin capable to stimulate production of tissue cytokines. The aim of this work was to determine the impact of β-alanine on humoral immune response in low-dose allergy model. BALB/c mice were immunized by recombinant Asp f 2 protein or commercial ovalbumin (OVA) in the withers 3 times a week with or without β-alanine supplementation. To determine the mechanism of β-alanine effect, α-L-alanine, an isomer which is not MrgD receptor ligand, and β-aminoisobutyrate with β-alanine-like affinity to MrgD ligand, were compared. According to our data, β-alanine stimulated specific IgE and IgG1 production in a short-term course (7 immunizations) and enhanced antibody affinity after long-term (14 immunizations) protocol in the case of low-immunogenic protein Asp f 2. In the case of high-immunogenic OVA protein, the impact of β-alanine was significant only upon antibody affinity. Hence, β-alanine accelerates specific IgE production in the case of low-immunogenic protein. The impact of β-alanine on specific IgE production was not linked to specific MrgD receptor activation, because β-aminoisobutyrate, which is the other ligand of this receptor, did not have a similar effect upon humoral immune response. The effect of β-alanine on IgG1 production seems also independent of MrgD receptor, since the common proteinogenic amino acid α-L-alanine also enhanced specific IgG1 production. The effect of β-alanine on humoral immune response could be linked to its non-specific action, e.g., due to its ability to induce oxidative stress through blocking taurine transporter, or due to its ability to stimulate cellular metabolism.

Keywords: β-alanine, IgE, low doses, antibody affinity, immunogenicity, MrgD receptor

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Introduction

Currently the hypothesis about the key role of alarmins released from damaged tissue cells in triggering pro-allergic immune response is becoming more widespread [12, 18]. Tissue cell damage may occur, in particular, during parasitic invasion [1, 12] or in response to protease allergens [12]. It is well known that after the invasion of macroparasites (mainly helminthes) into the organism, activation of IgE production and type 2 immune response generally occurs. So the concept according to which the main physiologic function of specific IgE production and type I hypersensitivity reaction is to triggering the mechanisms that kills macroparasite in host organism appeared [1]. Alarmins could be either high-molecular substances for example HMGB1 proteins [5] or low-molecular components such as ATP [11] or uric acid [10]. Alarmins stimulate tissue cytokines production interleukins (IL) 25, 33 or thymic stromal lymphopoetin (TSLP) in neighboring cells [10, 11, 12, 27, 34], which in turn triggers IL-5 and IL-13 production [25, 29] and also though more infrequent IL-4 production [19] in type 2 lymphoid cells. The latter in turn activates specific IgE production and type 2 immune response [2, 30].

According to Medzhitov's hypothesis [20] much more substances could act as alarmins or DAMPs (Danger-associated molecular patterns). For example, neurotransmitters, which are responsible for itch sensing [20]. Among these substances β-alanine attracts special attention because it is not only an itch neurotransmitter [9] via its specific MrgD receptor [32] but also potential classic type alarmin because it is present not only in specific nerve endings but also in skeletal muscle fibres (in the form of easy hydrolysable dipeptides carnosine and anserine) [4]. It is known that muscle fibers as often as epithelial cells

are damaged upon helminthes migration through tissues [1]. It should be mentioned also that β-alanine and β-aminoisobutyric acid are the end products of pyrimidine nucleotides catabolism [16], intensification of which is very likely occurs after necrotic tissue cells damage and/or after DNA release in extracellular medium. According to the recent data the effect of a widely used adjuvant Alum is based on the triggering of DNA release from dying cells [15]. Though authors consider extracellular DNA itself as an alarmin [15], the effect of the products of its catabolites cannot be entirely ruled out.

In our previous work we have shown that β-alanine is capable to stimulate tissue cytokines production [8] and so probably stimulates pro-allergic immune response.

The aim of this work was to determine the impact of β-alanine on allergen-specific immune response in our recently developed low-dose allergy model [7].

Materials and methods

Materials

β-alanine, β-aminoisobutyric acid, α-L-alanine and ovalbumin (grade V) were obtained from Sigma Aldrich (Darmstadt, Germany). Tween-20 was obtained from AppliChem (Darmstadt, Germany). NaCl, Na_2HPO_4, NaH_2PO_4, KCl, used for washing buffer preparation were obtained from DiaM (Moscow, Russia). Bovine serum albumin (BSA) for ELISA blocking buffer preparation was obtained from Serva (Heidelberg, Germany).

Animals

BALB/c mice were obtained from "Andreevka" (Pushino, Russia) and were housed in conventional conditions during 12-hour light-dark cycle and *ad libitum* feeding. All manipulations were carried out according to IBCh RAS IACUC protocol.

Recombinant Asp f 2

Recombinant Asp f 2 protein (from *Aspergillus fumigatus*) with C-terminal His-Tag was expressed in E. coli M13 cells and was purified with Ni-NTA resin based chromatography as described in [24]. The resulting protein concentration was measured by Bradford method (kit from ThermoScientific).

Immunization and sample collection

BALB/c mice were immunized 3 times a week during 5 weeks with ovalbumin (OVA) or Asp f 2 in different experiments with 100 ng/mice/injection dose in withers site without any stimulus or with β-alanine (0,26 or 2,6 mg), α-L-alanine (2,6 mg) or β-aminoisobutyric acid (BAIBA) (2,6 mg) in total volume of 0,1 ml. Some animals were immunized with physiological saline for control.

Blood was taken by retro-orbital bleeding under isoflurane anesthesia 2 days after 7th (16th day) or 14th (32th day) immunizations. Serum samples were obtained by incubation of collected blood for 20 minutes at +37 °C and subsequent centrifugation at 600 g for fibrin cloths separation.

ELISA

Microtiter plates (Costar Maxisorb, USA) were incubated with 50 μl/ well solution of OVA or Asp f 2 in PBS (pH = 7,2) 5 μg/ml for specific IgG_1, IgG_{2a} or high affinity IgE measurement or 20 μg/ml for low affinity IgE measurement overnight at +4 °C. Between stages plates were washed with washing buffer (0,05% Tween-20 in PBS). Serum samples were titrated in blocking buffer (5% BSA in PBS for specific IgE measurement or 1% BSA in all other cases). Biotin-labeled anti-mouse IgEa (UH297, BioLegend), anti-mouse IgG1 (RMG1-1, BioLegend) and anti-mouse IgG2a (RMG1-2a, BioLegend) were used as primary antibodies. Streptavidin-HRP conjugate (BioLegend) was used in subsequent stage. 3,3',5,5'-tetramethylbenzydine was used as a substrate. Optical density was measured at 450 nm with substraction of 620 nm value as background with MultiScan FC automatic reader (ThermoScientific, USA). Serum titre was estimated as serum dilution in which optical density was equivalent to background optical density plus 3 its standard deviations.

Statistics

Statistics data processing was performed with Mann-Withney test. Mean and standard deviations were calculated.

Results

Impact of β-alanine on Asp f 2 specific humoral response

The impact of β-alanine on Asp f 2 specific humoral immune response was studied by administration of this substance in the withers. Allergen was administrated in low dose because we have previously shown that these doses induce more selective IgE production [7]. Data from Figure 1 A-C indicates that after short-term immunization protocol (7 immunizations) β-alanine significantly and substantially enhances Asp f 2 specific IgE production. Production of specific IgG1 but not IgG2a was also enhanced by β-alanine. In the same time after long term immunization protocol (14 immunizations) when specific antibody titres reach a maximum values specific IgE and IgG_1 production were not increased by β-alanine addition (Figure 1D-F). Therefore, β-alanine accelerates IgE production.

Impact of β-alanine on OVA specific humoral response

To verify the above-described data the same experiment with another antigen commercial OVA was performed. The results indicate that β-alanine stimulated only specific IgG1 but not IgE production. After 14th

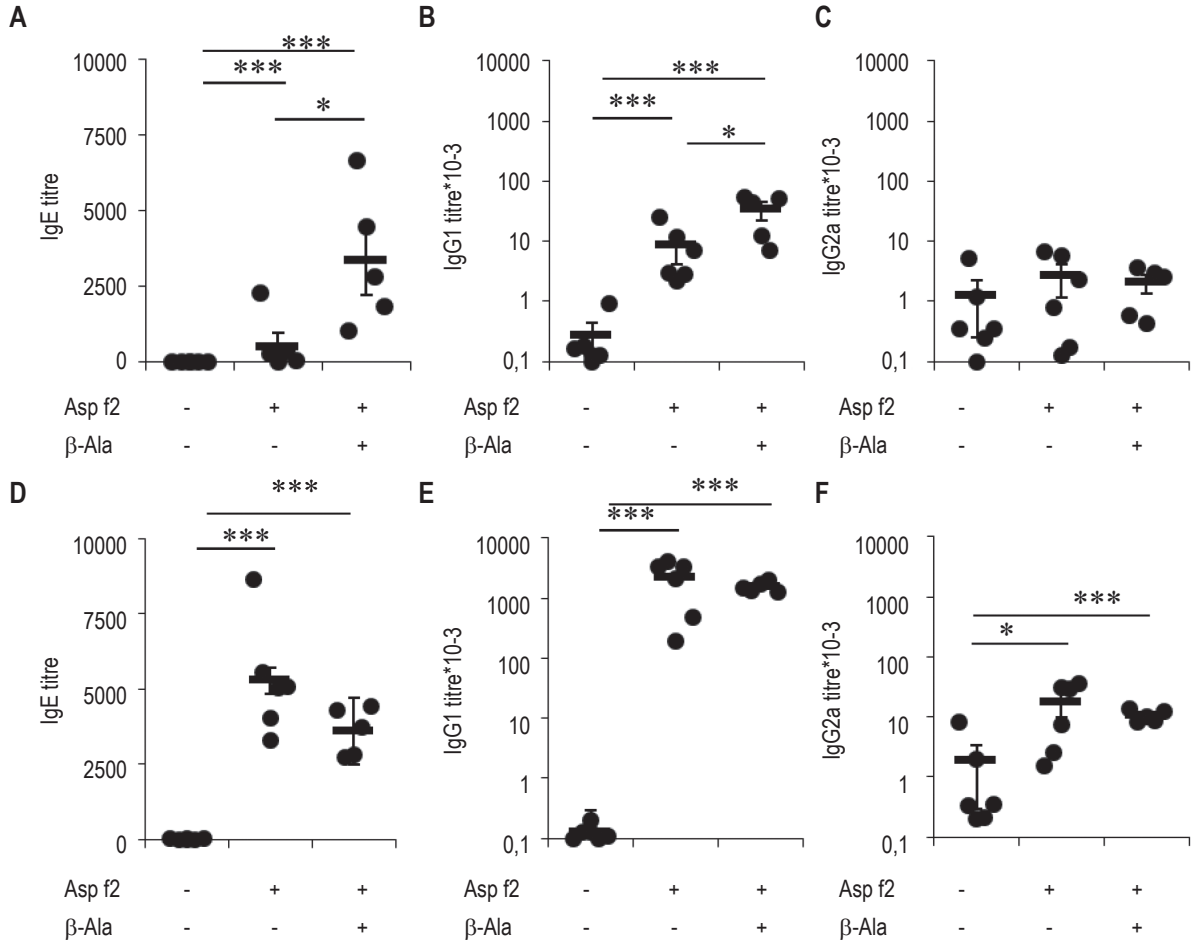

Figure 1. β-alanine accelerated formation of Asp f 2 specific IgE and IgG1 antibodies production but did not enhanced their final production and did not influence specific IgG2a production

Note. Titres of Asp f 2 specific IgE (obtained with coating buffer concentration 20 μg/ml) (A, D), IgG1 (B, E) and IgG2a (C, F) in BALB/c mice after 7th (16th day) and 14th immunization (32th day) by recombinant protein in 100 ng dose with or without β-alanine in comparison to control (physiologic saline immunized) mice. Data obtained in two independent experiments, mean values and standard deviations are shown. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

immunization there was no effect on specific IgE and IgG1 titers (data not shown).

Besides from relative quantity of specific antibodies their affinity is also a very important parameter. It is well known that the resulting affinity of antibodies which bind antigen epitope or specific hapten on ELISA microwell plate depend on a relative epitope concentration per unit area and the method of high- and low-affinity anti-hapten antibody measurement is based on this fact [21]. Due to mostly conformational nature of IgE epitopes [13, 22] the use of haptenilated protein in our case was outside of our purpose. Therefore, for estimation of high-affinity IgE antibodies fraction we carried out by ELISA method with the relatively low antigen concentration in coating buffer, exactly 5 μg/ml instead of 20 μg/ml for low affinity IgE. Indeed, in this case the effect of β-alanine on OVA specific IgE production was significant (Figure 2A). It is interesting that when the same coating buffer protein concentration was applied in the case of Asp f 2 we could could detect specific IgE binding only at the lowest serum dilution 1:10 in contrast to the situation with high coating buffer concentration.

When titrating monoclonal antibodies tangents of the slope of titrating curve are directly proportional to antibody affinity and for the polyclonal sera they will be directly proportional to the mean affinity of individual clones [3]. Concerning this fact Asp f 2 affinity was estimated as titration curve slopes in the coordinates lg(serum dilution coefficient)- optical density. Results showed that β-alanine increased mean Asp f 2 specific IgE antibody affinity (Figure 2B).

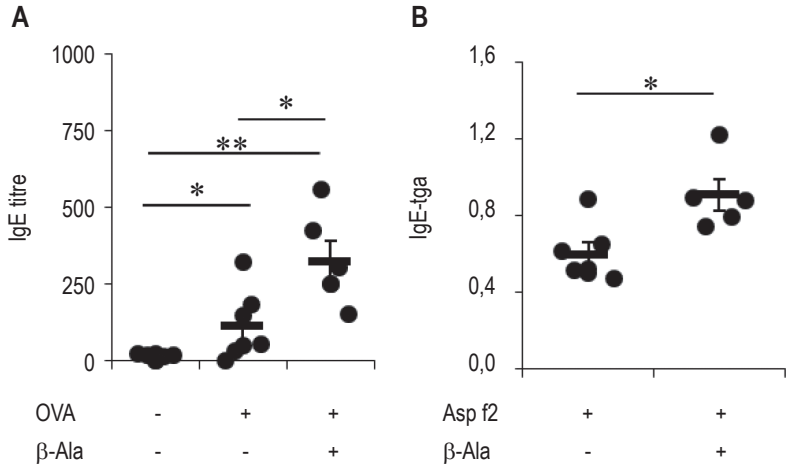

Figure 2. β-alanine enhances high affinity IgE production

Note. Titres of OVA specific IgE in BALB/c mice after 14th imminizations with OVA in withers site in 100 ng dose with or without β-alanine obtained in ELISA with coating buffer concentration 5 μg/ml (A) and slopes of Asp f 2 specific IgE titration curves in coordinates lg(serum dilution) – optical density, taken with the opposite sign, for serum samples of BALB/c mice after 14th immunizations with Asp f 2 without and with β-alanine (B). Data obtained in two independent experiments, mean values and standard deviations are shown. *, p < 0.05; **, p < 0.01.

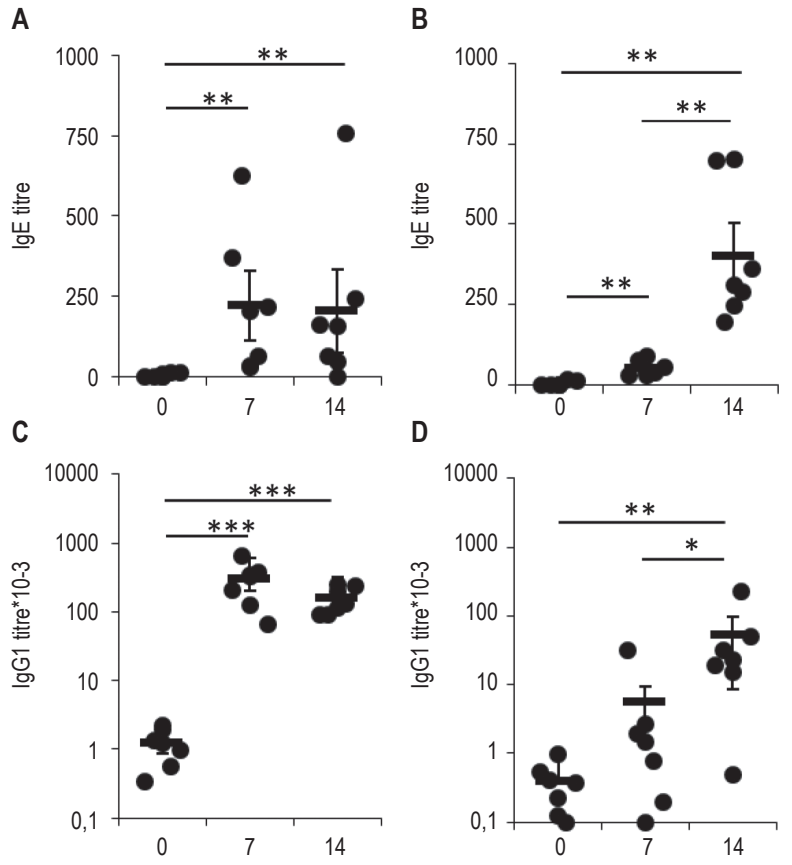

Figure 3. Different rate of OVA and Asp f 2 specific antibody response formation in the same mice

Note. Titres of OVA specific (A, C) and Asp f 2 specific (B, D) IgE (A-B) and IgG1 (C-D) antibodies in intact BALB/c mice (0), after 7th (7) and 14th (14) immunizations in withers region with OVA + Asp f 2 antigen mixture in 100 ng dose each. Data obtained in two independent experiments, mean values and standard deviations are shown. *, p < 0.05; **, p < 0.01; ***, p < 0.001.

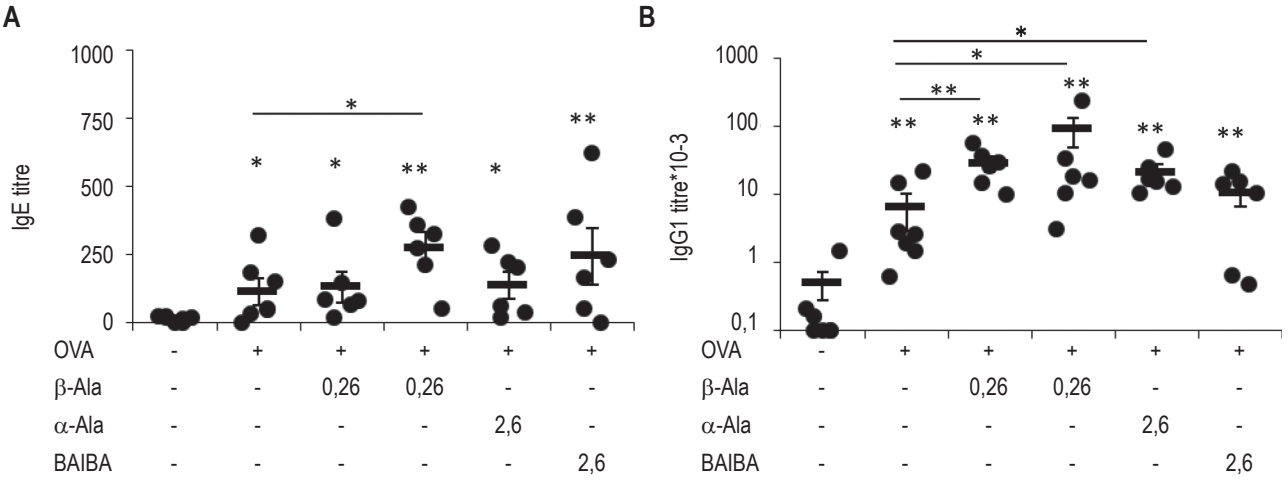

Figure 4. Impact of β-alanine on IgE and IgG1 production is not linked with MrgD receptor activation

Note. High affinity specific IgE (obtained in ELISA with coating buffer concentration 5 μg/ml) (A) and IgG1 (B) after 14th immunizations (32th day) by OVA in 100 ng/dose in withers site in BALB/c mice with or without indicated stimulus. α-Ala, alpha-L-alanine; β-Ala, beta-alanine; BAIBA, β-aminoisobutyrate. Data obtained in two independent experiments, mean values and standard deviations are shown. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Comparison between OVA and Asp f 2 specific humoral immune response

The differences in the effect of β-alanine on the humoral response could be explained by the different immunogenicity of the two proteins for animals. To verify this hypothesis we immunized mice with the mixture of two proteins OVA and Asp f 2 (100 ng dose each). We have shown that OVA specific IgE production appears earlier (after 7th immunizations) then Asp f 2 specific IgE production (after 14th immunizations) (Figure 3). Moreover, OVA specific IgE and IgG_1 production did not change at two time points. And thereby, the differences of the β-alanine impact on humoral immune response in the case of these two proteins could be due to a combination of two facts: different immunogenicity of these two proteins for animals and the nature of this antigens, more precisely acceleration but not increase in general allergen-specific immune response during long-term immunization.

The impact of β-alanine is manifested only at high concentrations and is not associated with MrgD receptor

It is well known that substances such as amino acids, which could be easily included in metabolic pathways and bind to specific transporters after administration in high concentrations, could influence physiological processes by this way and not through their specific receptors. To understand whethre the impact of β-alanine could be due to the activation of its specific MrgD receptors or by other non-specific ways we have performed experiment with α-L-alanine (common proteinogenic amino acid and β-alanine isomer that does not activate MrgD) and β-aminoisobutyric acid (other non-proteinogenic amino acid that bind and activate the same receptor as β-alanine [32]). Also in one additional group β-alanine was administrated in a dose 10 times lower (0,26 mg) than in previously experiments. If the impact of β-alanine is linked with MrgD activation it must appear with much lower administrating dose because of relatively high affinity of MrgD to β-alanine ($Kd = 10^{-8}M$) [9, 32] and also with administration of β-amiboisobutyrate but not α-L-alanine.

In fact, the increase of specific IgE production was found only after administration of high dose of β-alanine but not β-aminoisobutyrate. Low β-alanine dose did not show the same impact and in the case of α-L-alanine it was a week non-significant tendency. Moreover, both β-alanine in both doses and α-L-alanine, but not β-aminoisobutyrate increased specific IgG1 production (Figure 4). According to these data, the effect of β-alanine on specific IgE production was not linked to MrgD receptor activation.

Discussion

Alarmins like ATP or uric acid, which are released from damaged epithelial cells or proteases, could be the main stimulus of tissue cytokines production in early phases of pro-allergic immune response [5, 10, 11, 12]. We have previously shown that β-alanine, the substance specific to muscle tissue [16] and released from myocytes after their damage, could activate tissue cytokines production [8]. It is also important that β-alanine is an itch mediator [9] and the end-product of DNA pyrimidine bases catabolism [16]. Itch often accompany allergic inflammation [20, 31], and the release of DNA from cells with its subsequent deg-

radation is often occur after cell necrosis [15] which may be linked to macroparasite invasion [1].

In these work we have shown that β-alanine appears as an adjuvant of type 2 allergic immune response due to its enhancing effect on specific IgE and IgG1 but not IgG2a production. The impact of this substance appears in different ways when using proteins with different immunogenic potential for experimental animals and is not linked with activation of its specific MrgD receptor. Nonetheless above described phenomena may be of interest because its allows to get close to the understanding of the mechanisms which regulate specific IgE production. In the case of low-immunogenic protein Asp f 2 the impact of β-alanine appears on the levels of specific IgE produced after short-term immunization and affinity of these antibodies after long-term immunization, but in the case of high-immunogenicity protein OVA the impact was significant only on the affinity of producing antibodies.

Literature data obtained in genetically engineered mice where IgE production was coupled with fluorescent protein expression showed that specific IgE production in germinal centers occured during very limited time and that IgE^+ B-cells in germinal centers probably did not pass affinity selection to the same extent as for $IgG1^+$ B-cells [28, 33]. It is interesting that according to some recent results at least in some cases Ig antibody class switching could occur outside germinal centers during extrafollicular antibody response [17, 23]. It is also known that in early stages extrafollicular B-cells in comparison to germinal center B-cells have higher natural affinity B-cell receptor [6]. But extrafollicular foci exist for a short time (up to 2 weeks) [14] and it is logical to suppose that β-alanine supports formation of these structures. Further investigations are needed to be carried for refinement of this fact.

In the same time the impact of β-alanine appears only after its high dose administration and since this impact does not appear after administration of another MrgD ligand β-aminoisobutyrate [32] it is not linked to MrgD receptor activation. Moreover also α-L-alanine, a conventional proteinogenic amino acid, did not stimulate specific IgE production in our work while it stimulated specific IgG1 production. So it may be that in the case of β-alanine its impact on specific IgE and IgG1 production may be implemented by different mechanisms.

One of the probable mechanism of β-alanine action which could explain its action after high but not low dose administration is the activation of oxidative stress in tissue and (or) immune cells due to its ability to compete with antioxidant taurine for specific transporter [26].

Despite β-alanine effect only after high administrated dose and the ambiguity of this effect (the influence only on high affinity antibody fraction when using high-immunogenicity protein) the obtained data let us get closer to the understanding of the potential mechanisms regulating IgE production.

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