HUMORAL RESPONSE TO EPSTEIN-BARR VIRAL INFECTION IN PATIENTS WITH ALLERGIES

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Type I hypersensitivity is mediated by the production of IgE antibodies in response to normally harmless substances. Debate still continues about the mechanisms underlying allergic reactions. Reduced barrier tissue function can be one of the risk factors for allergies. The aim of the present work was to compare the humoral immune response to Epstein-Barr virus in patients allergic to the *A. alternata* fungus or *D. farinae* house dust mites and healthy donors. It is known that up to 90% of the world population are infected with EBV. This infection occurs at early age when a child develops allergy. The antibodies were analyzed using immuno-PCR and the recombinant EBV protein rEBNA. We were able to demonstrate that infection occurs at early age in both allergic patients and healthy donors. The proportion of EBP-seropositive individuals was comparable between the groups (75 and 74%). The proportion of patients with high IgG_1 titers among patients with allergies was lower (7%) than in healthy donors (18%), suggesting a lower viral load. In patients with allergies (but not in healthy donors) IgG_1 titers declined as children grew older (p = 0.037). Besides, IgA_1 titers were increased in patients with allergies in comparison with healthy donors, but differed between patients allergic to *A. alternata* and house dust mites. In allergic individuals, production of IgM against EBV was triggered earlier than in healthy donors. We conclude that IgM production and the IgA_1-mediated humoral response occur earlier in patients with allergies, causing a decline in IgG_1 titers over time.

Keywords: allergy, house dust mites, *A. alternata*, recombinant allergens, Epstein–Barr virus, immuno-PCR

Author contribution: Svirshchevskaya EV measured IgE titers against allergens in the sera of allergic individuals and healthy donors, processed the obtained data and participated in the writing of this article; Khlgatian SV selected sera samples for the study and conducted RIDA assays; Fattakhova GV and Chudakov DB measured IgE titers against allergens in the sera of patients with allergies and healthy donors; Matushevskaya EV collected sera samples from patients with allergies, participated in the discussion of the study results and in the writing of this article; Simonova MA and Ryazantsev DYu performed iPCR; Ryazantsev DYu expressed recombinant proteins of EBV, *A. alternata* and *D. farinae*; Chudakov DB synthesized, purified and characterized the sufficient amount of recombinant proteins for the study; Zavriev SK optimized PCR and participated in the discussion of the study results.

Compliance with ethical standards: the study was approved by the Ethics Committee of Mechnikov Research Institute of Vaccines and Sera (Protocol 35 dated September 12, 2018).

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Received: 12.09.2018 **Accepted:** 15.02.2019 **Published online:** 01.03.2019

DOI: 10.24075/brsmu.2019.004

ГУМОРАЛЬНЫЙ ОТВЕТ НА ВИРУС ЭПШТЕЙНА–БАРР ПРИ АЛЛЕРГИИ

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Аллергия I типа опосредована формированием IgE-антител к безвредным веществам. Механизмы возникновения аллергии остаются дискуссионными. Одним из факторов риска может быть снижение защитных функций барьерных тканей. Целью работы было проанализировать гуморальный иммунный ответ на вирус Эпштейна–Барр (ВЭБ) у больных с аллергией на гриб *A. alternata* и клещей домашней пыли *D. farinae* (КДП) и у здоровых людей. Известно, что до 90% населения инфицированы ВЭБ. Инфицирование происходит в раннем возрасте параллельно с формированием аллергических реакций. Анализ антител проводили методом иммуно-ПЦР с использованием рекомбинантного белка ВЭБ rEBNA1. Показали, что инфицирование как у больных, так и у доноров происходит в детстве; доля сероположительных по ВЭБ индивидов была сравнимой в группах (75 и 74%). Доля пациентов с высокими титрами IgG_1 среди больных с аллергией была ниже (7%) по сравнению с донорами (18%), что соответствует меньшей вирусной нагрузке. У больных с аллергией, но не у здоровых людей, наблюдали снижение титров IgG_1 с возрастом (p = 0,037). Кроме того, при аллергии повышены титры IgA_1 по сравнению с донорами, однако IgA_1-ответы при аллергии на гриб *A. alternata* и на КДП различались. При аллергии также раньше формировались IgM к ВЭБ. Таким образом, при аллергии быстрее формируется IgM и IgA_1 гуморальный ответ, что приводит к снижению с возрастом IgG_1-титров.

Ключевые слова: аллергия, клещи домашней пыли, грибы *A. alternata*, рекомбинантные аллергены, вирус Эпштейна–Барр, иммуно-ПЦР

Информация о вкладе авторов: Е. В. Свирщевская — определение IgE титров на аллергены в сыворотках больных с аллергией и доноров, обработка данных, написание статьи; С. В. Хлгатян — подбор сывороток больных с аллергией и доноров различного возраста, типирование методом RIDA; Г. В. Фаттахова, Д. Б. Чудаков — определение IgE титров на аллергены в сыворотках больных с аллергией и доноров; Е. В. Матушевская — сбор сывороток больных с аллергией, обсуждение результатов и написание статьи; М. А. Симонова, Д. Ю. Рязанцев — постановка ПЦР; Д. Ю. Рязанцев — экспрессия в *E. coli* рекомбинантных белков ВЭБ, *A. alternata* и *D. Farinae*; Д. Ю. Чудаков — наработка, очистка и характеристика рекомбинантных белков; С. К. Завриев — оптимизация ПЦР, участие в обсуждении результатов.

Соблюдение этических стандартов: исследование одобрено этическим комитетом Института вакцин и сывороток им. И. И. Мечникова (протокол № 35 от 12 сентября 2018 г.).

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Статья получена: 12.09.2018 **Статья принята к печати:** 15.02.2019 **Опубликована онлайн:** 01.03.2019

DOI: 10.24075/vrgmu.2019.004

The Epstein–Barr virus (EBV) is a DNA virus that belongs to the *Herpesviridae* family and causes a broad range of pathologies in humans, from respiratory diseases to cancer. So far, 8 herpesvirus types are known that infect humans. Among them, herpes simplex virus (types 1 and 2), varicella zoster (type 3), EBV (type 4), and cytomegalovirus (type 5) are widely spread in the human population. In contrast, infections caused by types 6 and 7 herpesviruses and Kaposi sarcoma-associated herpesvirus are much rarer. Almost every adult is infected with at least one type of herpesvirus. The diagnosis is established based on the presence of specific antibodies in the blood serum. About 80 to 95% of the world population are latently infected with EBV or cytomegalovirus [1]. Latent EBV infection is associated with some cancers [1–3], multiple sclerosis [4–5], and systemic lupus erythematosus [6]; it also aggravates the course of HIV infection [7] and triggers production of autoantibodies against human DNA and proteins [8–9]. The main EBV antigens are the viral capsid antigen, the early antigen and the nuclear antigen 1 (EBNA1) [10–11]. EBNA1 ensures persistence of the virus in its latent state. Type G antibodies (IgG) against EBNA1 are produced by the organism every time the virus reactivates and reflect the total body viral load.

EBV is spread through bodily contacts, such as kissing, sharing personal hygiene items, eating utensils or the like. Airborne transmission is quite rare though possible. Mother-to-child transmission occurs during pregnancy, childbirth or breastfeeding. According to some researchers, antibodies against EBV are detected in 50% of children under 3 years of age [12–13]. In such cases, the virus is likely to be spread through sharing eating utensils and kissing.

Type I hypersensitivity is characterized by a IgE-mediated humoral response to the proteins contained in small, normally harmless particles, such as pollen, house dust mites (HDM), animal dander, etc. [14–15]. The skin and bronchial epithelium of patients with allergies differ considerably from barrier tissues of healthy individuals [16–17]. The aim of the present study was to measure a humoral response to EBV in patients allergic to *A. alternata* and *D. farinae*.

Previously, we proposed a method for measuring IgG_1 titers against EBV and other allergens based on the quantitative polymerase chain reaction (PCR) [18–19]. Immuno-PCR (iPCR) is a sensitive technique that can detect antibodies in biological fluids [20–21]. Its advantage is the linearity of titration curves in a wide range of concentrations, which enables detection of specific antibodies using a smaller number of dilutions [18–19].

METHODS

Sera

Serum samples used in the present study were collected from children and adults with hypersensitivity to HDM and the *Alternaria alternata* fungus at Mechnikov Research Institute of Vaccines and Sera (Moscow, Russia) between 2009 and 2017. Informed consent was obtained from all donors or their representatives. Allergy tests were performed using commercial RIDA panels (Germany). The samples collected from patients with allergies were included in the study if IgE titers against *A. alternata* or *D. farina* measured by RIDA were interpreted as classes 3 through 6. Patients who had previously undergone allergen-specific immunotherapy or had cross-sensitivity to *A. alternata* or *D. farinae* were excluded from the study. Individuals of different ages who had no IgE against pollen, fungi or domestic allergens included in the RIDA panel were considered healthy donors.

Materials

The following reagents and equipment were used in the study: high protein-binding capacity well-plates (Costar; USA), Nunc TopYield strips for qiPCR (ThermoFisher Scientific; USA), Tween-20, a ready-for-use 3,3'5,5'-tetramethylbenzidine solution (Sigma; USA), goat serum (Bovogen Biologicals; Australia), biotinylated monoclonal anti-human IgG_1–IgG_4 and anti-IgE antibodies (Southern Biotech; USA), mouse anti-human IgA_1 and IgA_2 antibodies, a conjugate of goat anti-mouse IgG and biotin and a conjugate of streptavidin and horseradish peroxidase (BD Pharmigen; USA), biotinylated oligodeoxyribonucleotide (ODN) (Lumiprobe; Moscow), streptavidin (Sigma; USA) and recombinant proteins rEBNA1, Der f 2 and Alt a 1 synthesized in our laboratory [18, 22–23]. Other reagents were purchased from Fluka (Switzerland).

Immuno-PCR

The 10 μkg/ml solution of the recombinant rEBNA1 antigen in carbonate-bicarbonate buffer (0.05 M, pH 9.6) was pipetted into the TopYield wells (50 μl per well) and incubated overnight at 4 °C. In the morning the wells were washed with TETBS (20 mM Tris-HCl, 150 mM NaCl, 0.1 mM EDTA, 0.1% Tween-20, pH 7.5) three times. Serum samples were diluted tenfold with TETBS containing 20% goat serum, and a series of 1 : 5 dilutions was prepared for each sample. Each diluted sample was pipetted into the well-plates (25 μl per well) in three replicates. Six replicates of fetal bovine serum (FBS) were used as a negative control. The plate with the samples was incubated on the shaker at room temperature for 30 min. Then, the plate was washed with TETBS three times. Solutions of biotinylated anti-human IgG_1, IgG_2, IgG_3, and IgG_4 antibodies or mouse anti-human IgA_1, IgA_2 and IgM antibodies in TETBS containing 20%-goat serum (1 : 1,000) were introduced into the wells (50 μl per well). The plates were incubated on the shaker at room temperature for 30 min and then washed three times with TETBS. To measure IgA_1, IgA_2 and IgM concentrations, the samples were further incubated with goat anti-mouse IgG antibodies conjugated to biotin. After washing, 50 μl of 1 μkg/ ml streptavidin were introduced into the wells, incubated for 10 min, and washed. Then, 50 μl of 5 pM ODN solution in TETBS containing 20% goat serum were added into each well and incubated on the shaker at room temperature for 10 min. After incubation, the wells were washed 3 times with TBS (20 mM Tris-HCl, 150 mM NaCl, pH 7.5). Thirty-five μl of the PCR mix were added in the wells and overlaid with 30 μl of mineral oil per well. Real-time PCR was performed in the DTprime thermocycler (DNA-Technology; Russia). Briefly, the protocol included initial 5-min denaturation at 94 °C followed by 40 cycles of annealing and extension at 60 °C for 15 s and denaturation at 94 °C for 5 s. For each cycle, fluorescence from the probe was recorded at 520 nm wavelength. PCR results were analyzed using the thermocycler software provided by the vendor. For each sample, a mean threshold cycle value (Cq) and a standard deviation were computed. The detection threshold was calculated as 3 standard deviations for (Cq-), where (Cq-) is a threshold cycle value in negative samples. The titers were determined as a maximal dilution of a serum sample at which the sample was positive for a measured analyte.

Statistical analysis

Mean and standard deviations were computed in Excel (Microsoft Office, 2003). The correlation between IgG1-antibody titers

and the groups of patients was evaluated using the parametric Pearson's χ2-test and Student's *t*-test. The differences were considered significant at $p < 0.05$ yielded by the two-tailed analysis.

RESULTS

Specifics of iPCR

The iPCR-based method used in this study is described in the Table. On the whole, steps 0-3 of iPCR are similar to those of ELISA: the antigen is applied onto a plate (step 0); incubated with a blood serum sample (step 1); then with biotinylated anti-human IgG1 antibodies (step 2); streptavidin-ODN (PCR) or a streptavidin-horseradish peroxidase conjugate (ELISA, step 3). Step 3 in iPCR is divided into two substeps and includes incubation with streptavidin followed by incubation with biotin-ODN, which enhances iPCR sensitivity. Step 4 is purely PCR or the addition of a substrate for horseradish peroxidase (ELISA).

In ELISA, the substrate-based detection step remains unstandardized and depends on the day of experiment and the time of reaction termination. Using iPCR, one can reduce the time required for the reaction by as much as 1 hour. The iPCR makes the analysis more standardized and therefore less dependent on the operator. It also increases the sensitivity of the analysis due to a stronger linearity of the obtained data [18–19].

IgE-mediated response to allergens

Serum samples collected from allergic individuals were assayed using commercial RIDA panels. Those containing IgE antibodies against *Dermatophagoides farinae* and *Alternaria alternata* were included in the initial phase of the study. Specifically, we selected the samples interpreted as class 3 and above, according to RIDA scores. Fig. 1 shows distribution of the allergic patients into groups based on RIDA classes. In some samples, no IgE antibodies were detected against any of 15 allergens present in the panel (pollens, fungi and domestic allergens). Such sera were used as healthy donor samples. Ultimately, a total of 30 samples were selected representing patients with allergies and healthy donors aged 0 to 15 years.

Total humoral response to EBV

A pool of 10 RIDA class 5 and 6 serum samples was used to profile the repertoire of anti-EBV antibodies in patients aged 3–15 years with respiratory allergies to HDM and *A. alternata*. A pool of samples collected from 10 healthy donors of the same age was used as a control. The titers of rEBNA1-recognizing immunoglobulins were as follows: IgM > IgG_1 > IgA_1 > IgA_2 > IgG_2 (Fig. 2) in donors and IgM > IgA_1 > IgG_1 > IgA_2 > IgG_2 in patients with allergies. The titers of IgG_3 and IgG_4 were low in both groups. Significant differences between allergic patients and healthy donors were observed only for IgG_1 and IgA_1. The IgG_1 to IgA_1 ratio was 9 and 0.4 for healthy donors and allergic patients, respectively, suggesting that the IgA-mediated response prevailed in the studied cohort of patients whereas the IgG_1-mediated response prevailed in healthy donors.

Analysis of IgG_1-mediated response to EBV

Viral infections normally trigger production of class IgG_1 antibodies. Fig. 3A shows age-based distribution of IgG_1 titers against EBV in the sera of patients allergic to HDM and/or *A. alternata* and healthy donors. The analysis revealed that infection had been acquired at early age in both groups. A few 4–6-year-old children in both groups had IgG_1 levels above 1,000. Mean IgG_1 titers were 330 and 1,500 in the group of patients aged 3 to 10 years and healthy donors, respectively. In patients aged 11–20 years and healthy donors of the same age, the titers were 720 and 490, respectively, indicating a tendency to early infection or early immune response. Because of the considerable variability in the data, no significant differences

Table. Comparison of iPCR and ELISA steps

Steps	0	1	2	3	4	
PCR	Antigen	Serum	aIgG1-bio*	Streptavidin/ODN	PCR	Time
	Overnight	30 min	30 min	10/10 min	1 h	3–4 h
ELISA	Antigen	Serum	aIgG1-bio	Streptavidin-HP**	Substrate	
	Overnight	1 h	1 h	1 h	20 min	4–5 h

Note: *aIgG1-bio represents any biotinylated antibody to any class or subclass of antibodies (G, A, E); **HP is horseradish peroxidase.

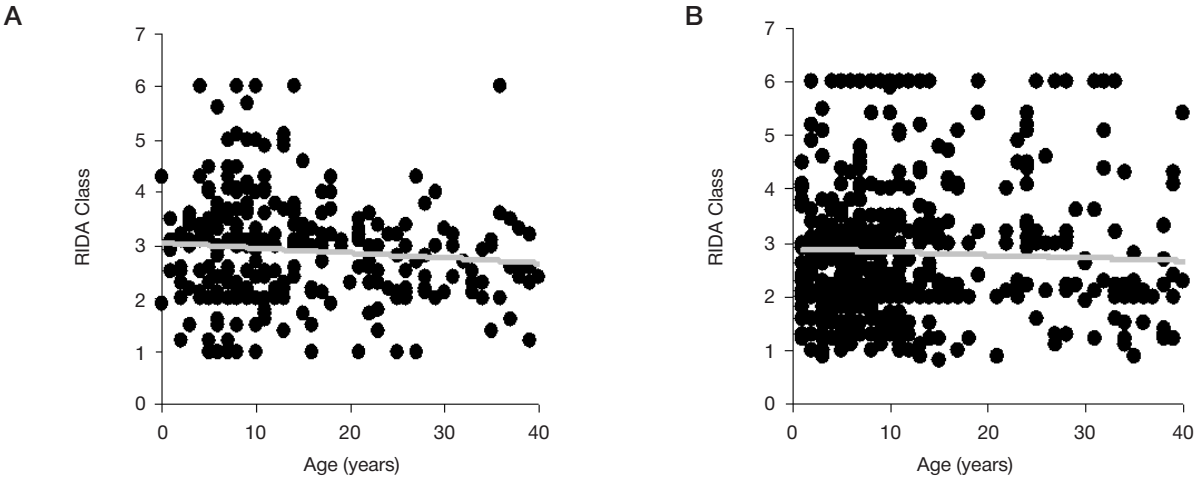

Fig. 1. IgE-mediated response in patients with allergies measured by RIDA. IgE levels in the serum samples of patients allergic to house dust mites (**A**) and *A. alternata* (**B**) were interpreted as classes 0–6 proportional to IgE titers

were observed between the groups. No differences were found in the level of antibodies against EBV between patients allergic to HDM or *A. alternata* and healthy donors (Fig. 3B).

While determining the proportions of individuals who did not have antibodies against EBV and those who had low (< 100), moderate (100–1,000) or high (>1,000) IgG_1 titers, we established that 75% of children in both groups aged 3–15 years had a latent EBV infection (Fig. 4A) and 45% of individuals in both groups had low titers of antibodies. The groups differed in terms of high IgG_1 titers: the titers over 1,000 (2,000–8,000) were observed in 20% of healthy donors and only 7% of allergic children (Fig. 4A). This suggests a better resistance to the viral infection in allergic patients. Anti-EBV antibody levels were comparable in both groups (Fig. 4B).

Analysis of IgA_1 and IgM-mediated responses to EBV

As shown above, IgA_1 antibodies were slightly though reliably increased in patients with allergies ($p = 0.03$). A more detailed analysis revealed that the most pronounced difference could be observed at early stages of the viral infection (Fig. 5A). Moderate anti-EBV IgA_1 titers in patients with allergies and healthy donors aged 3 to 10 years reached 425 and 265, respectively; in patients aged 11–30 years, they were 690 and 370, respectively. Besides, IgA_1 titers tended to increase with age in both groups (Fig. 5A). Interestingly, IgA_1 titers against EBV were indicative of the difference in response to the viral infection between patients with different allergies. The levels of IgA_1 against EBV were significantly higher in patients with allergies to HDM (Fig. 5B) than in patients with IgE against *A. alternata* and in healthy donors.

In both healthy donors and patients with allergies, moderate IgM titers against EBV were higher than class G and A immunoglobulin titers by one order of magnitude. The obtained data were split into two groups: low titers (< 5,000) and high titers (> 15,000) (Fig. 5C, D). The proportion of individuals with high IgM titers in both groups was ≈ 60%. No differences in IgM levels depending on the age and mean titer were observed in the high IgM subgroup (Fig. 5C). In the low IgM subgroup, patients with allergies produced anti-EBV antibodies earlier

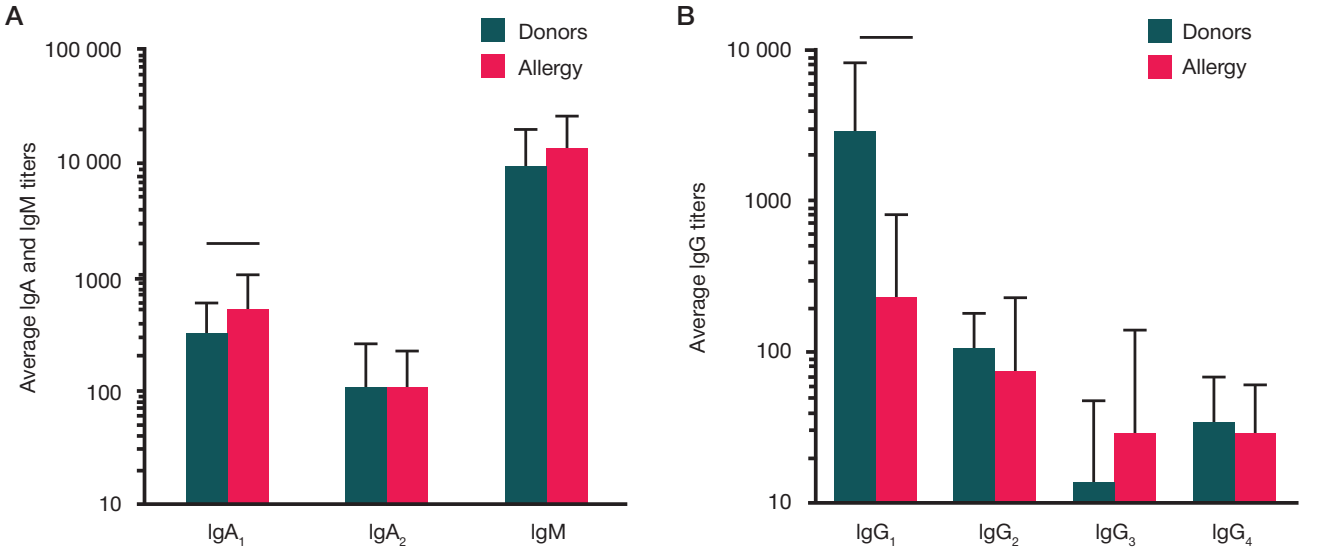

Fig. 2. Analysis of rEBNA1-recognizing immunoglobulins present in the sera of healthy donors and patients with allergies. Analysis of IgA_1, IgA_2, IgM (**A**), IgG_1, IgG_2, IgG_3, and IgG_4 (**B**) in the pooled sera of 10 donors and 10 allergic patients. The figures are presented as mean values ± a standard deviation. Statistically significant differences ($p < 0.05$) are marked with a vertical line with a cross-bar

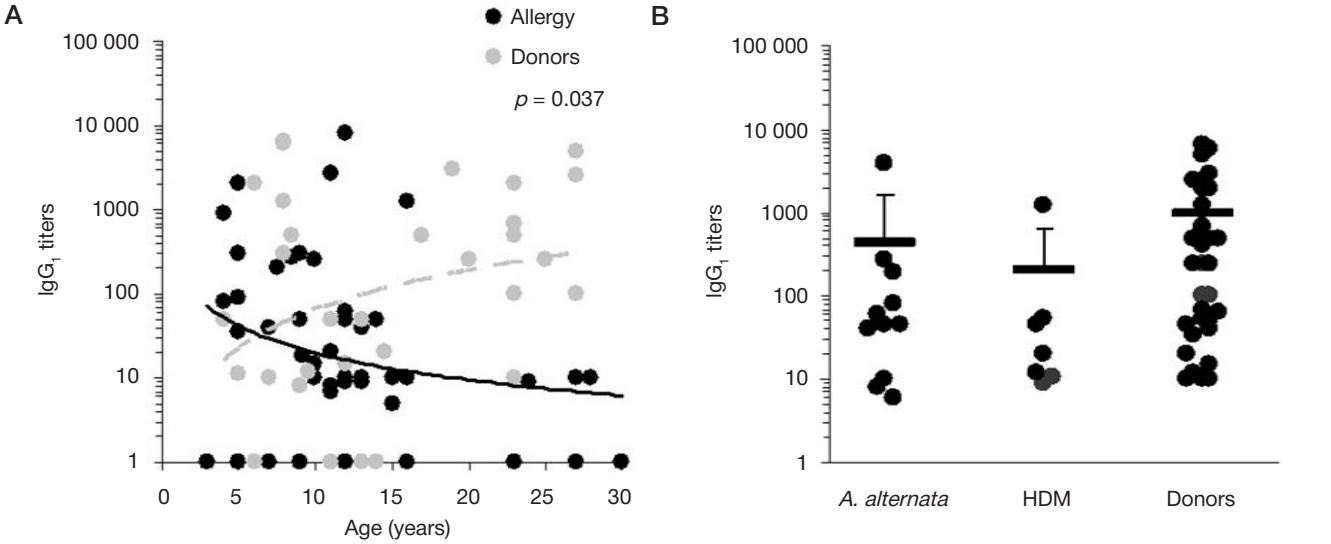

Fig. 3. Analysis of rEBNA1-specific IgG_1 in the sera of healthy donors and patients with allergies. **A.** Analysis of IgG_1 titers in individual sera of patients allergic to *A. alternata* ($n = 15$), house dust mites ($n = 9$) and healthy donors ($n = 38$). Vertical lines with cross-bars indicate mean values ± a standard deviation. **B.** Relationship between rEBNA1-specific IgG_1 titers and age in the individual samples of sera collected from allergic patients ($n = 53$) and healthy donors ($n = 34$). Power approximation is marked with lines and t-test probability is also shown

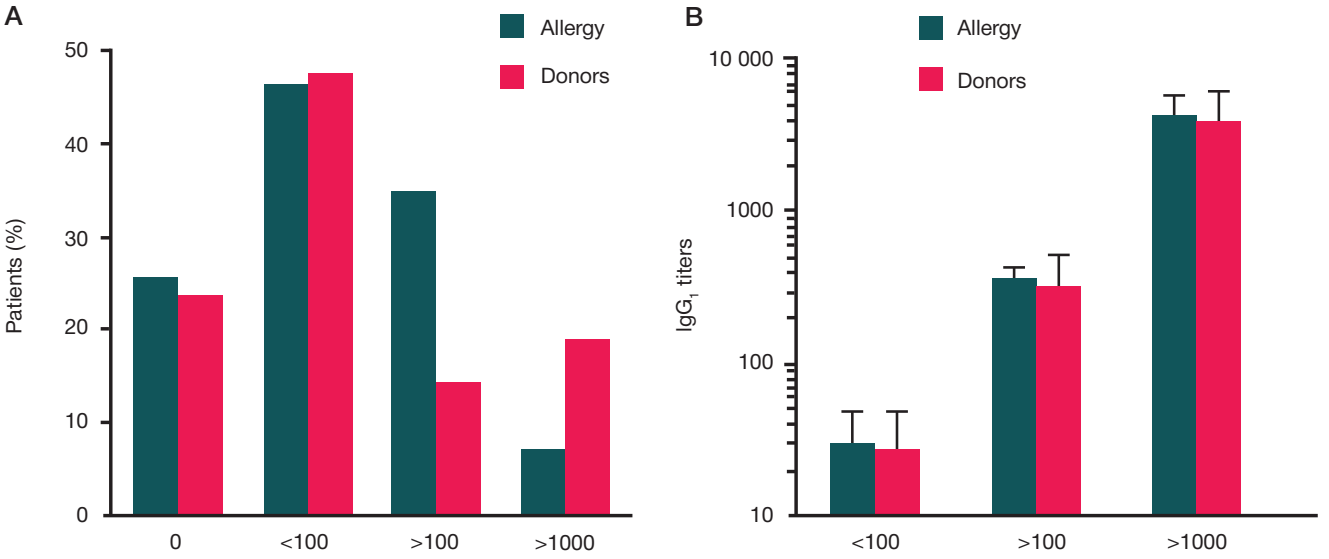

Fig. 4. Analysis of rEBNA1-specific IgG_1 present in the sera of healthy donors and patients with allergies. The proportion (%) of patients and healthy donors with different IgG_1 titers (**A**) and the mean titers in these groups (**B**)

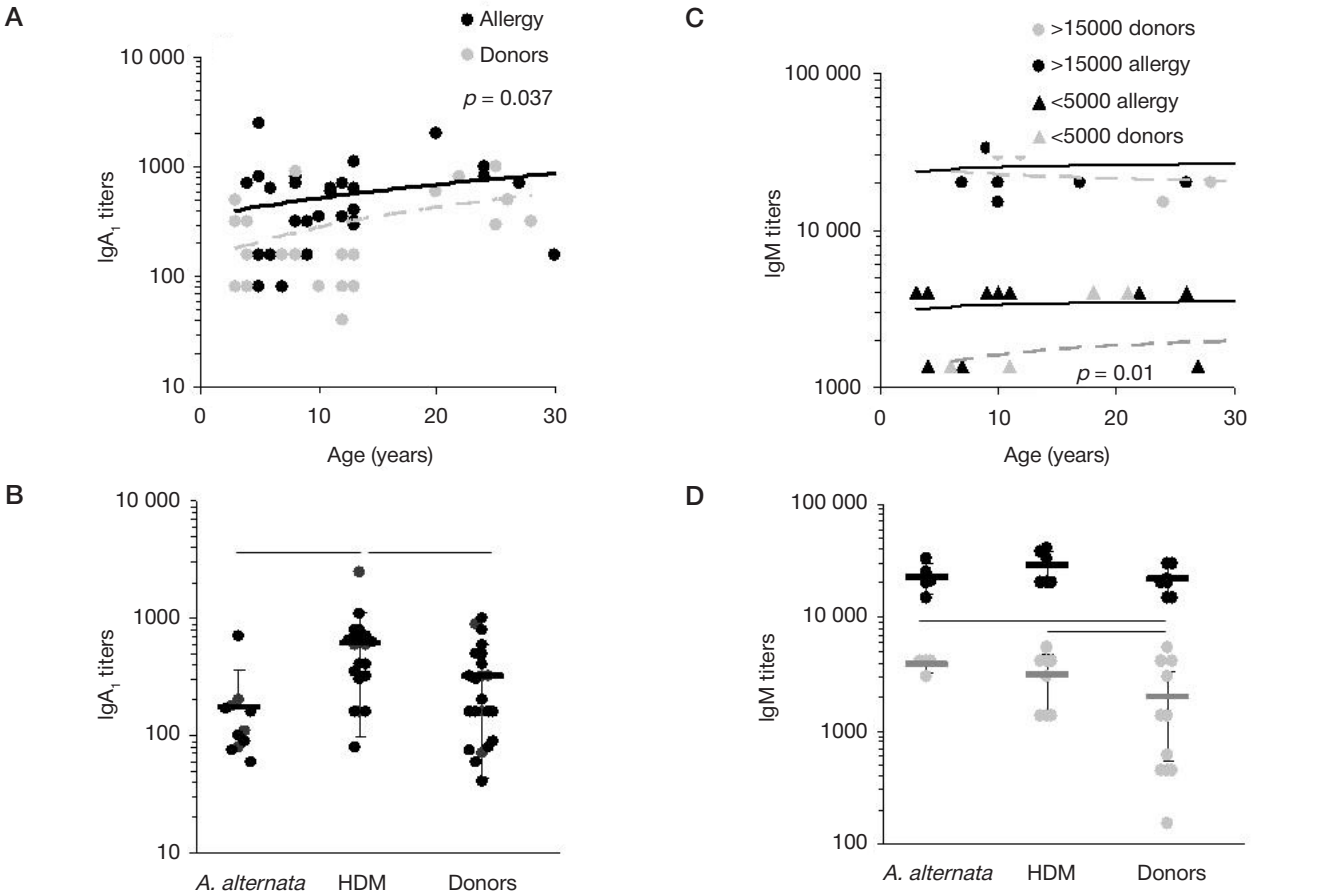

Fig. 5. Analysis of rEBNA1-specific IgA_1 and IgM in the sera of healthy donors and patients with allergies. Distribution of IgA_1 (**A**) and (**B**) titers in the individual serum samples of patients allergic to *A. alternata* (n = 11), house dust mites (n = 21) and healthy donors (n = 23) depending on donors' age. Distribution of rEBNA1-specific IgA_1 (**C**) and IgM (**D**) in the sera of healthy donors and patients with allergies. IgM data are given for the sera with low (grey circles) and high (black circles) IgM titers. Vertical lines with cross-bars show mean values ± a standard deviation. Significant differences ($p < 0.05$) are marked with horizontal bars

than healthy donors (Fig. 5D). A rise in IgM titers in the low IgM subgroup was detected in patients allergic to both HDM and *A. alternata* (Fig. 5D).

DISCUSSION

After primary infection with EBV, the organism starts to produce different (sub)classes of antibodies. A human body is capable of producing isotypes M, A, G and E that also include the IgG_1–IgG_4 and IgA_1–IgA_2 subtypes. The main pool of class M antibodies represents innate immunity; IgM titers increase during primary infection. IgA is involved in mucosal defense. IgE rises in response to parasitic infections and allergens. At present, it is believed that IgA and IgE are adaptive immunity components because their production requires B-cells to "switch" from secreting IgM to other immunoglobulins. However,

recently there has been a lot of debate about the possibility of such "switch" occurring without participation of T cells [23, 24], which is how innate immunity functions. Class A antibodies are produced in response to exposure to early antigens, such as VCA and EA [25]. IgA and IgM antibodies to early antigens are markers of viral reactivation or secondary infection. IgA titers against the late EBNA1 antigen are also significantly increased in patients with nasopharyngeal cancer [26].

The data yielded by our experiment demonstrate that patients with allergies responded to EBV infection by an early and significant increase in IgA_1 and IgM titers. Their IgA_2 titers were lower than IgA_1 and did not differ significantly between the groups (these data are not provided in the present article). Allergies are accompanied by mucosal inflammation and increased production of cytokines and chemokines [17, 24], leading to the activation of B cells. IgM production was comparable between healthy donors and allergic patients (both with a strong serological response) indicating an equally strong immune response to the reactivation of IgM-secreting B cells by the virus.

IgG proteins are the main protective component of the adaptive humoral response. The "switch" of B cells to IgG production occurs only in parallel with the antigen-specific T-dependent response to EBV. It is known that IgG1 secretion dominates antiviral response [27–28]. According to the literature, antibody titers produced in response to hepatitis B infection are as follows: $IgG_1 > IgG_4 > IgG_3 > IgG_2$ [27]; the pattern changes in the case of EBV: $IgG_1 >> IgG_2$, IgG_3, IgG_4 [28]. The data obtained for healthy donors are consistent with the EBV response pattern dominated by IgG_1 production. For patients with allergies, mean IgG_1 concentrations were lower than in healthy donors and comparable with IgG_2 concentrations ($p = 0.14$). Statistical analysis revealed that significant differences in IgG_1 titers between allergic patients and healthy donors increase with age. So far, the antiviral response in patients with allergies has not been studied. On the whole, the data yielded by our experiment suggest that respiratory allergy is accompanied by an increase in IgA_1- and IgM-antibodies against EBV, which prevents the virus from penetrating the epithelial barrier and reduces the total body viral load.

CONCLUSIONS

Allergy is characterized by hypersensitivity of epithelial barriers caused by an interaction between IgE and allergens. Hyperreactivity of the innate immune system seems to enhance the antiviral response to the Epstein-Barr virus, causes a rise in IgA_1 and IgM titers and a reduction in IgG_1 titers correlating to the latent viral load.

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