Age-Related Features of Mast Cell Reactivity in Allergies

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Abstract

Introduction: During last few years, a novel scientific sphere has developed and is successfully growing, associated with the study of age-related changes in immune and allergic reactions at the cellular level. Although there is a fact that mast cells are the subject of many researches, many aspects of their physiology and pathology remain unsolved. Moreover, clarification in the age-related features of mast cell reactivity makes a great contribution to the expansion of our view of point on the characteristics of the clinic, etiology and pathogenesis of allergies in ontogenesis.

Materials and Method: We used 443 guinea pigs of both sexes of different ages, previously experienced 10-14 day-quarantine control under conditions of vivariums. Animals were constantly monitored, body weight was periodically determined, and rectal temperature was measured. Sensitization and anaphylactic reactions were reproduced by conventional method. Normal horse serum was used as an allergen. The resolving dose of a specific allergen was administered once intravenously or intraperitoneally 14-16 days after the start of the first sensitizing dose of the allergen. Collected digital data processed on electronic microcalculator Rokwell international Model 44 (USA).

Results: With age, the rate of filling mast cells with granules increases. In guinea pigs of an older age, as compared to newborns, the rates of filling mast cells with granules are 1.3-2.2 times greater ($p < 0.05$), which is consistent with the literature. Spontaneous mast cell degranulation was observed in intact animals. Their frequency ranged from 5.4 to 8.6% and, in principle, did not depend on age. A comparative analysis of the frequency of degranulation, depending on age, showed that the frequency of degranulation in sensitized newborns is slightly lower than in older animals. However, with age in sensitized guinea pigs, the frequency of degranulated mast cell forms increased and the mast cell reactivity in one month aged pigs became approximately the same as in mature animals.

Conclusion: In general, the functional activity of mast cells increases with age, and by the age of one month reaches the level of functional activity. In the development of specific therapy, a decrease in the titer of homocytotropic antibodies and the percentage of mast cell degranulation is vital.

Keywords: *Allergy,mast cells, sensitization, degranulation, allergens, age-related features.*

Introduction

During the life of organ systems of human body, namely, homeostasis, protective and adaptive reactions, metabolic processes, blood circulation, respiration and others, an important role is played by connective tissue and blood. Among various connective tissue cells, mast cells are particular and learnt with high interest by scientists (Nasonov, 2017)[1]. They accomplish

extremely important functions: participate in the regulation of homeostasis, secrete biologically active substances (heparin, histamine, serotonin, dopamine, etc.), affect the entire system of connective tissue, and are actively involved in allergic reactions. In this regard, during the recent years, a new scientific field has arisen and is successfully developing, associated with the study of age-related changes in immune and allergic reactions at the cellular level (Baglay et al, 2015)[2]. For a long time, the prevailing opinion in medical science was that cells are always damaged as a result of the formation of an immune complex on the surface of their membranes (Tsibulkina et al, 2017)[3]. It has now been clearly established that the formation of immune complexes on the surface of target cells is not always accompanied by cell destruction (Gushchin, 2019)[4]. In most cases, it is not destruction, but excitation, that is, an increase in their functional activity. This information opens up a new promising area that allows us to develop pharmacological method for the management of allergic reactions and not it is possible to use drugs that reduce and inhibit excitatory processes in mast cells (Kryshen et al, 2018)[5].

Despite the fact that mast cells are the subject of many researches, many aspects of their physiology and pathology remain unsolved[6]. So, for example, the question of age-related features of mast cell reactivity in normal and allergic reactions is of great scientific interest (Artishevsky et al, 2016). The fact is that mast cells are among the most reactive cells in the body. They quickly respond to various exogenous and endogenous effects (Namazova-Baranova et al, 2017)[7]. The study of mast cell reactivity in the dynamics of allergy development allows us to determine the pathological process in the early stages of their development, which is of great diagnostic, therapeutic and preventive value. It is believed that the clarification of the reactivity of mast cells allows one to indirectly judge the general condition of the organism as a whole. In addition, mast cell reactivity can be used to objectively evaluate the effectiveness of therapy (Abaturov et al, 2017)[8].

Thus, elucidation of the age-related features of mast cell reactivity contributes to the expansion of our ideas about the features of the clinic, etiology and pathogenesis of allergies in ontogenesis[9]. Above mentioned points make us to conduct this research. The identification of age-related features of mast cell reactivity in allergic reactions, in our opinion, will contribute to the successful solution of certain fundamental aspects of the National Program of the Ministry of Health of the Republic of Uzbekistan for the treatment and prevention of allergies[10].

The aim of the study was to identify age-related features of mast cell reactivity in allergic conditions and to develop criteria for therapeutic effectiveness based on an experimental model of specific hyposensitizing immunotherapy[11].

The age-related features of reactivity, quantitative content, filling of cells with granules, functional activity of tissue (mesentery) and peritoneal mast cells in intact guinea pigs have been established[12]. It was revealed that the body of guinea pigs in early postnatal ontogenesis (newborns, two weeks) is reactive to exogenous allergenic effects[13]. It was determined that the reactivity of mast cells of guinea pigs increases with age and reaches a level of reactivity in mature adults. It was established that mast cells of young guinea pigs are actively involved in the development of allergic reactions of an immediate (anaphylactic) type[14].

Criteria have been developed for evaluating therapeutic efficacy based on an experimental model of specific hyposensitizing immunotherapy in guinea pigs of different ages[8]. It has been established that the most important objective indicators of experimental specific therapy of anaphylactic allergy are the determination of the level of HCA titer in blood serum, the frequency of mast cell degranulation and the anaphylactic index[3].

Materials and Method

Study Population: The experiments were conducted on 443 guinea pigs of both sexes of different ages, which previously passed 10-14 day-quarantine control under conditions of vivariums: experimental - 274, control - 169. By age, guinea pigs were distributed as follows: newborns - 108 (24.4%), two-weeks - 117 (26.4%), one-month - 120 (27.1%), mature - 98 (22.1%).We paid attention to the general condition, feed intake, mobility, shine of the coat, absence of outflows from visible mucous membranes (eyes, nose, anal and genital openings). Animals were constantly monitored, body weight was periodically determined, and rectal temperature was measured. Animals were fed with normal (traditional) food. Before the experiments animals were not fed during one day. In general, we provided with all the necessary requirements for experimental research. Sensitization and anaphylactic reactions were reproduced by conventional method[1].

Normal horse serum was used as an allergen. The resolving dose of a specific allergen was administered once intravenously (hind paw) or intraperitoneally 14-16 days after the start of the first sensitizing dose of the allergen. The severity of general anaphylactic shock and the calculation of the anaphylactic index (AI) were evaluated by Weigle et al. The histamine shock is reproducible in two versions: 1) the deadly histamine shock was reproduced by intravenous administration of 0.1% histamine at the rate of 4-6 mg/kg of animal body weight; 2) non-fatal histamine shock was reproduced by subcutaneous administration of histamine at the rate of 2 mg/kg of animal body weight[2].

Homocytotropic antibodies (HCA) in the blood serum of sensitized guinea pigs are determined by the generally accepted method of reproducing a cutaneous passive anaphylactic reaction. The reaction was considered positive if the diameter of the stained spot on the skin was at least 6 mm. Tissue (mesentery of the small intestine) and peritoneal mast cells in guinea pigs were obtained by a well-known method in compliance with sterility.

Mast cells were fixed in absolute alcohol and stained with 1% solution of toluidine blue in 60° alcohol. The frequency of granular mast cell forms showed in percentage by viewing different fields of view of smears under a microscope with a magnification of 5x95. Specific therapy was carried out according to the scheme developed by us. To control animals, sterile saline was injected subcutaneously at the same time instead of a specific allergen.

Statistical Data Analysis: Digital data processed on electronic micro calculator Rokwell international Model 44 (USA). The following indicators were calculated: 1) arithmetic and geometric mean values; 2) the negative logarithm of the geometric mean titer of antibodies when the base is equal to two; 3) the correlation coefficient; 4) the significance of differences between the compared average values. Information was considered reliable provided that t22, and $p < 0.05$.

Results

An analysis of our own experimental studies revealed the age-related features of mast cell reactivity in normal and in allergic reactions, anaphylactic type. In intact guinea pigs, mast cells are scattered in all organs and systems of the body. However, their quantitative content in tissues compared with other animal species (rats, mice, hamsters) is much less.

Nevertheless, in the development of allergic reactions of guinea pigs, they are crucial (Eliseeva et al, 2016). In this regard, it was of some interest to clarify the age-related characteristics of the number of mast cells in guinea pigs. In the mesentery of the small intestine of intact newborn guinea pigs (with an increase of 100 times), the number of mast cells, in comparison with guinea pigs, is more than that of older age (two-weeks, one-month, sexually mature) for 1.9-3.7 times (Table 1), which is consistent with the literature.

It is believed that this phenomenon is associated with a deep change in the environment after birth, mechanical influences during childbirth, etc. According to some researchers, the decrease in the number of mast cells after birth is explained by the blockade of the color of the protein-polysaccharide interaction, which is more important for the proliferation of mast cells. The analysis of mast cell fullness by granules was observed with great interest; where biological active substances are concentrated (histamine, serotonin, etc.).

Guineapigsage	The number of mast cells in the field of view (M±m)	Mast cell degranulation rate (M ± m%)
Newborns (n=10)	268,9±11,2	5,4±1,6
Twoweeks (n=10)	136,0±6,2*	6,2±1,3
One month (n=10)	84,1+1,1*	7,6±0,6
Sexuallymature (n=10)	72,7+2,2*	8,6+0,7
Total: 40	---	---

Note: * The difference in comparison with the performance of newborns is significant ($p < 0.05$).

They determine the pathochemical phase of the pathogenesis of an allergy of an immediate type. The indicator of filling mast cells with granules is given great importance because a smaller filling with granules does not indicate that they are poorly developed, but, on the contrary, increase their functional activity. According to our data, age-related features of filling mast cells with granules are observed in intact guinea pigs. With age, the rate of filling mast cells with granules increases. In guinea pigs of an older age, as compared to newborns, the rates of filling mast cells with granules are 1.3-2.2 times greater ($p < 0.05$), which is consistent with the literature.

Thus, on the whole, the following regularity was revealed — in guinea pigs of different ages: in newborns, in comparison with older animals, the total number of mast cells is greater, and the nature of filling the cells with granules is less.

According to our data, spontaneous mast cell degranulation was observed in intact animals. Their frequency ranged from 5.4 to 8.6% and, in principle, did not depend on age (table. 1). However, the age-related features of mast cell degranulation in allergic reactions were clarified. Sensitization showed a significant increase in the mast cell degranulation rate, in comparison with similar indicators in intact guinea pigs, 1.2-3.0 times ($p < 0.05$). A comparative analysis of the frequency of degranulation, depending on age, showed that the frequency of degranulation in sensitized newborns is slightly lower than in older animals. However, with age in sensitized guinea pigs, the frequency of degranulated mast cell forms increased and the mast cell reactivity in one month aged pigs became approximately the same as in mature animals (Table 2).

Table 2: Age-related features of the frequency of degranulation of mast cells of the mesentery of the small intestine in guinea pigs with sensitization and anaphylactic shock (n = 145).

Age of animals	Sensitization (M± м%)	Anaphylactic shock (M± m%)	Control (M± м%)
Newborn	10(6,5+0,8)	21(15,2+0,8)*	6(5,3+1,3)
Two weeks	10(12,3+0,9)**	19(27,2+1,3)*	6(6,0+1,1)
One month	10(25,6±1,3)**	20(61,0±1,6)*	6(8,0+0,7)
Sexually mature	10(21,9+2,3)**	21(65,0+2,1)*	6(7,3+0,7)
Total	**40**	**81**	**24**

Notes: Between indicators of anaphylactic shock and control * (*). and also between the sensitization and control indicators (**) reliability ($p < 0.05$).

The study of the reactivity of guinea pigs of different ages with experimentally reproduced anaphylactic shock was also of some interest. In the literature, there is conflicting information on this issue. Some researchers believe that in the early postnatal age, the body does not respond to allergenic effects, asimmunocompetent system is poorly developed. Other researchers believe that newborns are actively sensitized. It turned out that guinea pigs in early postnatal ontogenesis are reactive to exogenous allergenic effects, as evidenced by the development of general anaphylactic shock with parenteral administration of a resolution dose of a specific allergen. Along with this, age-related features of anaphylactic shock were revealed: in young animals (newborns, two weeks old), cases of mild and moderate severity of anaphylactic shock (AI = 1.7-2.6) prevailed, and in one-month-old and mature ones, severe and fatal (AI = 2 9-3.7). In the mechanism of development of allergic reactions in general and anaphylactic shock in particular, mast cells and their biological active substances are crucial. In anaphylactic shock, the frequency of mast cell degranulation in comparison with similar indicators in sensitization was significantly ($p < 0.05$) higher in newborns - 2.3 times, two-week 2.2 times, one-month 2.3 times, and mature - 2.9 times. Similar results were noted in the study of reactivity of peritoneal mast cells sensitization and anaphylactic shock (table. 3). With sensitization, compared with intact guinea pigs, the mast cell degranulation rate was 1.6 times higher in newborns in two weeks, 3.4 times higher one-month - 5.7 times, sexually mature - 7.2 times (p-0, 5). With anaphylactic shock, the frequency of mast cell degranulation was even greater than with sensitization.

Table 3: Age-related features of the frequency of peritoneal mast degranulation in guinea pigs during sensitization and anaphylactic shock (n = 81).

Age of the animals	Sensitization (M± м%)	Anaphylactic shock (M± m%)	Control (M± м%)
Newborn	12(10,7+0,97)**	12(13,5+0,88)*	6(6,5:0,96)
Two weeks	15(22,2+0,37)**	15(26,4+1,11)*	6(6,5.0,96)
One month	16(39,8:0,70)**	16(50,8+0,70)*	6(6,8.0,98)
Sexually mature	14(45,8+1,4)**	14(73,9+2,43)*	6(6,3+0,7)
Total	57	57	24

Note: the differences between anaphylactic shock and control (*), as well as between sensitization and control, are significant ($p < 0.05$).

Discussion

In the mechanism of development of anaphylactic shock in guinea pigs, the decisivehistamine is important (Soldatov et al, 2016). According to our data, with histamine shock, the frequency of mast cell degranulation increased sharply, moreover in older animals, which indicates a sharp increase in the functional activity of these cells. In histamine shock, the mast cell degranulation rate in sexually mature guinea pigs was 4 times higher than the mast cell degranulation rate in newborn guinea pigs.

The question arises: why in rats, mice and hamsters, where there are many times more mast cells than guinea pigs, the clinical symptoms of anaphylactic shock are weakly expressed? Apparently, this is due to the fact that their mast cells are scattered in all tissues and organs more or less evenly. In addition, immunocompetent cells of these animals weakly synthesize humoral antibodies (Johansson et al, 2001). In the body of guinea pigs, the spread of mast, cells around the smooth muscles of the bronchioles, bladder and intestines is much greater than in other animals. In this regard, during allergic reactions on the cytoplasm of mast cells of guinea pigs, a large amount of histamine is released, which causes spasm of bronchioles, contraction of the smooth muscles of the gastrointestinal tract and bladder, and expansion of peripheral small arteries and arterioles (Reese et al, 2002). As a result of this, all the main symptoms of anaphylactic shock acutely develop: asphyxia, hypoxia, involuntary defecation and urination. One of our tasks was to study the importance of mast cells in the mechanism of development of specific therapy. Although specific therapy has been used in practical medicine for more than 90 years, nevertheless there are many unresolved issues, among which age-related features of indications and contraindications, the importance of mast cells in the mechanism of development of specific therapy, are important.

Studies have shown that specific HCA are detected in the blood serum of sensitized animals. However, the HCA titer depended on the age of the experimental animals: fewer (3.5 ± 1.2) y newborns and more (7.5 ± 0.6) in older guinea pigs. After specific treatment, the HCA titer in animals of all age groups, compared with the HCA titer before treatment, decreased 1.7-2.3 times ($p < 0.05$). The study of mast cell reactivity with specific therapy showed the following results. The frequency of mast cell degranulation compared with indicators before treatment significantly decreased ($p < 0.05$). Specific therapy also effectively prevented the development of general anaphylactic shock. All animals remained alive. Excellent results were observed in the sexually mature (AI = 0), good results were observed in the one-month (AI = 0.1), two-week (AI = 0.3) and newborns (AI = 0.6). Analysis of the actual material indicates that mast cells also take an active part in the mechanism of development of specific therapy, along with cells of the immunocompetent system.

In general, currently, there are various theories regarding the pathogenesis of specific therapy: increasing the synthesis of blocking antibodies, neutralizing specific allergic antibodies, switching the synthesis of allergic antibodies to the synthesis of blocking antibodies, strengthening the factors of nonspecific defense of the body, strengthening the synthesis of anti-inflammatory factors, etc.

All these theories are based on actual clinical and experimental facts (Nadein et al, 2012). Our data support the theory of neutralizing specific allergic antibodies.

Indeed, according to our data, after specific therapy, the titer of specific HCA significantly decreased in all age groups of animals, including newborns, two-week and one-month-old ones. However, the mechanism of this phenomenon is not clear. There is an opinion that when conducting specific therapy, the process of multifaceted "microshocks", that is, the interaction of HCA with allergens, occurs. Thus, allergic antibodies are gradually eliminated. However, not all scientists agree with this treatment. It is believed that inhibition of HCA synthesis is crucial in the mechanism of specific therapy. Our data are also consistent with the theory of inhibition of the synthesis of mediators by mast cells. According to our data, there is a significant decrease in the frequency of mast cell degranulation in all age groups of animals. Thus, using a complex of allergological and immunological method, it was possible to identify age-related features of the reactivity of tissue and peritoneal mast cells, both in norm and in allergic reactions of anaphylactic type.

Conclusion

The following age-related features of tissue (mesentery of the small intestine) mast cells in intact guinea pigs are noted: their quantitative content in newborns is greater in 1.9-3.2 times, and the filling rate of cells with granules is 1.3-2.2 times less than that of two-weeks, one-month and sexually mature ones; functional activity of some (minimum) partmast cells of all age groups of guinea pigs is increased, as evidenced by the presence de granulated cell forms; in general, the functional activity of mast cells increases with age, and by the age of one month reaches the level of functional activity, the characteristics of the reaction are fat and mature.

Furthermore, the organism of guinea pigs in early postnatal ontogenesis (newborns, two weeks) is reactive to exogenous allergic effects, as evidenced byidentification of specific homocytotropic antibodies in the blood serum of sensitized animals and the development of anaphylactic shock with repeated parenteral administration of a specific allergen, with mild and moderate cases predominate in young people (AI = 1.7 and 2.6),and in one-month-old and sexually mature - heavy and fatal (AI = 2.9 and 3.7). The functional activity of tissue and peritoneal mast cells significantly increases in all age groups of guinea pigs, and with active sensitization in1.2-3.0 times, anaphylactic shock - 2.8-8.9 times, histamine shock - 3.0-10.6 times.An experimental model of specific therapy with a positive therapeutic effect can be successfully reproduced on young guinea pigs(newborns, two weeks, one month). In the mechanism of development of specific therapy, a decrease in the titer of homocytotropic antibodies and the percentage of mast cell degranulation is essential.

Recommendations: It is fact that the indicators of the frequency of mast cell degranulation, in combination with other clinical and laboratory data, can be used to objectively assess the initial state of an allergic reactive organism, which will improve the quality of specific diagnosis of allergies in the early stages of its development, and also when clinical symptoms are absent, that is, with latent sensitization. An experimental model of a specific hyposensitizing immunoterium FDI in guinea pigs of different ages can be used to evaluate the therapeutic efficacy of different allergens non-infectious origin. The discovery of new scientific facts about the active participation of mast cell mast cells in early postnatal ontogenesis during sensitization and allergic reactions broadens our understanding of the age-related features of the pathogenesis of immediate (anaphylactic) allergy in developing organisms.

1. The age-related features of reactivity, quantitative content, filling the cells with granules and reactive tissue (mesentery of the small intestine) and peritoneal mast cells in intact guinea pigs are noted.
2. The reactivity of mast cells of guinea pigs increases with age and reaches a level of reactivity in mature adults.
3. The organism of guinea pigs in early postnatal ontogenesis (newborns, two weeks) is reactive to exogenous allergenic effects. As evidenced by the successful reproduction of sensitization and anaphylactic reactions.
4. The mast cells of young guinea pigs (newborns, two weeks) take an active part in the development of allergic reactions of the immediate type, as evidenced by a significant increase in their functional activity during sensitization, anaphylactic and histamine shock.
5. In young guinea pigs, an experimental model of specific hyposensitizing immunotherapy is successfully reproduced, in the mechanism of development of which a decrease in the titer of homocytotropic antibodies and the percentage of mast cell degranulation is essential.

Study Limitations: The study is animal based research. Expected outcomes and results of the interventions might be different on humans. Thus, in case of implementation the achievements of the study on patients, this factor has to be taken into account.

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Ethical Approval: During the study respected the principles of a human attitude towards laboratory animals. The ethical approval for the study was granted by the Committee of Ethical Approval for Researches under the Ministry of Health of the Republic of Uzbekistan.

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References

1. Baglay E.O. DAI. “Mast cells are key participants in the pathogenesis of immunoinflammatory diseases”. Rheumatology science and practice. 2015; 53(2): p. 182-189.
2. A TVNaTN. “Mast cell as a multifunctional element of the immune system”. Allergology and Immunology in Pediatrics. 2017; (2 (49)): p. 4-11.
3. I.S. G. “Allergy is a late product of the evolution of the immune system”. Immunology. 2019; 40 (2): p. 43-57.
4. Kryshen K.L. KAE, MAA, MMN, & MVG. “Regulatory and methodological aspects of the study of the allergenic properties of new drugs at the stage of preclinical studies”. Vedomosti Scientific center for the examination of medical devices. 2018; 8(1): p. 44-55.
5. Namazova-Baranova L.S. SMAea. “Features of the diagnosis of allergies in children”. Immunology. 2017; 37(4): p. 64-78.
6. Eliseeva T.I. & II. “Allergic reactions to drugs: current understanding (review)”. Modern technologies in medicine. 2016; 8 (1): p. 159-172.
7. S.N A. “Clinical problems of drug allergy”. Medical News. 2016; (11 (266)): p. 41-45.
8. E.L. N. “New opportunities for pharmacotherapy of immuno-inflammatory rheumatic diseases: focus on interleukin inhibitors 17”. Scientific and practical rheumatology. 2017; 55 (1): p. 68-86..
9. Abaturov A.E. & AA. “The development of the immune response in staphylococcal pneumonia (part 1). Child Health. 2017; 12 (2): p. 200-210.
10. Soldatov A.A. AI, & NV. “Immediate-type allergic reaction mechanisms, drugs and specific immunotherapy method”. Immunology. 2016; 37 (1): p. 51-60.
11. Johansson S.G. HJO, BJ. “A revised nomenclature for allergy. An EAACI position statement from the EAACI nomenclature force”. Allergy 56. 2001;: p. 813 - 824.
12. C BJ. “Standardization of double-blind, placebo-controlled food challenges”. Allergy. 56. 2001; 67.: p. 75-77.
13. Reese I. WM. Stellenwertpseudoallergischer Reaktionen und Einflus von Zuckerbei der atopischen Dermatitis”. Allergologie. 2005;(2): p. 264-268.
14. Nadein KA. “Mast cells as a factor in the development of inflammatory processes in the connective tissue”. Clinical Pharmacology and Drug Therapy Reviews. 2012; 10 (1): p. 22-27.