An immunoregulatory role of nitric oxide (NO) in the development of adaptive immune responses associated with allergic diseases is very important. The present study extended these observations by the examination of the reciprocal changes in characteristic immunologic parameters of the disease and NO level of bronchoalveolar lavage (BAL) cells under guinea pig model of acute asthma with multiple challenges. Development of guinea pig Th2 mediated asthma was accompanied by increasing the level of allergic markers: ovalbumin (OVA) specific IgG and IL-4. We demonstrated that the infiltrate of airway cells contributes to NO synthesis in the respiratory tract during allergic inflammation. The level of intracellular NO formation significantly correlated with plasma allergen specific IgG value in OVA-induced asthma. The presented data evidence that the elevated intracellular NO level in BAL fluid may reflect a nitrosative stress in respiratory tract in general, when allergic asthma exacerbation is present.

Keywords: ovalbumin-induced asthma, sensitization, allergic inflammation, nitric oxide, bronchoalveolar lavage.

Asthma, a chronic inflammatory airway disease, is associated with bronchial hyperreactivity, tissue remodelling, and reversible airflow obstruction, which leads to recurrent coughing and dyspnea [1]. In general, asthma includes allergic, non-allergic and intrinsic phenotypes. Clinically, allergic asthma is characterized by allergen-induced, IgE-mediated early and late bronchial obstructive reactions, acute and transient airway hyperresponsiveness (AHR) after these reactions and infiltration of inflammatory cells, particularly eosinophils and Th2 type lymphocytes into the airways [2].

Increased generation of reactive nitrogen species is well documented in asthma [3, 4]. Though nitric oxide (NO) produced in the lungs is an important regulator of airway events, including modifying airway tone, regulating pulmonary vascular tone, stimulating mucin secretion, modulating mucociliary clearance [5]. Statement of NO impact presents an intractable challenge, since both protective and detrimental effects have been extensively reported [6-9]. Under the pathophysiologic process NO, a gaseous free radical, is generated in the respiratory tract by highly specialized cells of the respiratory epithelium, vascular endothelium and by genuine immune cells in response to inflammatory cytokines [6, 10, 11]. The detection of 3-nitrotyrosine in the lung of asthmatic patients provides definitive evidence for numerous biochemical targets of NO that are likely to have functional consequences in the asthmatic airways. The correlation between high exhaled NO level and eosinophilic-mediated airway inflammation in asthmatic patients has been substantially registered. Hence, measurement of fraction of NO in exhaled air (FeNO) is recommended as a baseline parameter of inflammation and corticosteroid responsiveness in conjunction with the traditional methods for diagnosing subjects with asthma [11, 12]. Thus, the recognition of NO level determination by international medical community as a valid noninvasive point-of-care tool for allergic airway inflammation evaluation is an irrefutable and hard fact.

Detailed understanding of the biochemical pathways that control NO metabolism and its physiological functions, will enable future rational design of strategies targeted to modulate abundant levels of NO products and consequently limit formation of more detrimental species. At this point in vivo models contribute substantially to issue solving.
However, no single model is sufficient to reproduce accurately a syndrome as complex as asthma to be [13]. Therefore, to address current knowledge gaps and to drive a fundamental shift in asthma research and drug development a tool-kit of clinically relevant approaches, that build a holistic picture of the disease, is to be elaborated. Here, we consider a model of acute asthma with multiple challenges following 32 weeks after sensitization, which mimics those seen in humans have been exposed to allergen a long period later acquired hypersensitivity. The current study aimed to identify intracellular NO generation under guinea pig model of acute asthma with prolonged period after allergen sensitization in order to examine the reciprocal changes in characteristic immunologic parameters of disease and NO level formed immediately by inflammatory immune cells infiltrating airways.

**Materials and Methods**

**Experimental design.** Specific pathogen-free (SPF) outbred male guinea pigs, weighing 300-350 g, were randomly assigned to 3 groups: (I) intact group (normal, n = 6); (II) sensitized animals (OVA/0.9%NaCl, n = 4); (III) allergic bronchial asthma group of sensitized animals with the allergen challenges (OVA/OVA, n = 6). The animals were housed at a temperature range of 22-24 °C under a 12 h light/dark cycle and were fed a regular diet. All procedures with animals were performed in accordance to requirements of General Ethical Principles of Experiments on Animals (Ukraine, 2001).

Sensitization was induced by virtue of the administration to albumin from chicken egg (OVA, Sigma Aldrich, USA) in conjunction with the adjuvant. Procedure was carried out by intraperitoneal injection (i.p.) of OVA (100 μg per animal) and Al(OH)₃ (Alfarus, Ukraine) (100 mg per animal) dispersed in 1 ml normal saline. Sensitization was reinforced by the second immunization of OVA (100 μg per animal) and Al(OH)₃ (10 mg per animal) in 1 ml saline 4 days later. The animals were used experimentally 32 weeks after sensitization.

Allergen challenges were carried out by inhalation of 0.1% OVA in normal saline for 5 times totally on days 1, 2, 3, 5 and 7. Provocation procedure was performed for up to 15 min in a transparent plastic cage (V = 5 l), in which the guinea pigs could move freely, coupled to compressor nebulizer. In order to counteract tolerance, the exposure duration was determined by the appearance of respiratory distress signs (sneezing, coryza, coughing, deep retraction of the thoracic wall and cyanose). With these conditions, none of the animals expressed anaphylactic shock resulting in lethal outcome. There are no animals which failed to develop the bronchial asthma clinical symptoms. The sensitized guinea pigs (OVA/0.9%NaCl) were challenged with aerosolized normal saline following the same schedule to exclude unspecific stimulation of the airways by the aerosol droplets. Suggested protocol reflects the allergen-driven pathway of asthma reproducing several characteristic features, such as airways infiltration by inflammatory cells, early (EAR) and late asthmatic reactions (LAR), AHR [14, 15] and wheezing in separated animals in the end of provocation schedule.

**Bronchoalveolar lavage fluid collection and blood processing.** Bronchoalveolar lavage (BAL) fluid samples were collected during LAR, at 18-20 h after the final challenge. Guinea pigs were anesthetized with thiopental sodium (190 mg/kg guinea pig weight i.p.) and administered lidocaine injection in the neck (8 mg/kg guinea pig weight subcutaneous injection (s.c.). The trachea was exposed and cannulated. The lungs were gently lavaged for 3 times with normal saline (1 ml/100 g guinea pig weight). Approximately 77% of the BAL fluid was retrieved. The returning fluid was centrifuged at 500 g for 15 min at 4 °C, BAL cells were resuspended in sterile normal saline. Total cell counts were determined using a Neubauer haemocytometer.

Blood was collected from Ear Veins to Eppendorf tubes containing heparin. Blood was centrifuged for 15 min at 1000 g within 30 min after collection. The samples were aliquoted and stored at -20 °C until assaying.

**BAL cell viability.** Briefly, 10⁶ washed cells per sample were used for ethidium bromide (EtBr, Sigma Aldrich, USA) staining. Since EtBr can penetrate inside the cells with integrity disturbance, it selectively labels DNA of dead cells in a mixed population. The fluorescent dye loading procedure lasted for 15 min at room temperature in darkness and afterwards samples were kept at 4 °C. The EtBr final concentration in incubation medium was 2.5 μM. Cell viability in suspension was assessed by flow cytometry technique.

**Immunological markers.** OVA specific IgG and IL-4 levels were measured in plasma using a commercial enzyme-linked immunosorbent assay kits (Cusabio, USA) following the protocols provided by...
the manufacturer. Absorbance spectra were determined spectrophotometrically by microplate reader µQuant (Biotek, USA) in the range of (380-600) nm with the step 5 nm. The absorbance maximum was set on 450 nm with the reference value at 570 nm. OVA specific IgG parameter was presented in conventional units (CU). Guinea pig OVA specific IgG content was considered positive when its absorbance value was higher than the index calculated from negative control absorbance: ODsample ≥ (2.1 x ODnegative) – positive; ODsample ≤ (2.1 x ODnegative) –negative. IL-4 content was expressed as picogram per milliliter (pg/ml) of plasma. The lower detection limit is defined as 0.78 pg/ml of IL-4 level.

**Intracellular reactive nitrogen species detection.** NO production in the living cells was monitored by measurement of triazole fluorescent derivative DAF-2T formed in the presence of NO and O2 from a non-fluorescent cell permeable 4,5-diamino-fluorescein diacetate (DAF-2DA, Sigma Aldrich, USA) [16]. BAL cell suspension was incubated with 10 µM DAF-2DA for 30-40 min at 37 °C in darkness. BAL cells (10^6) have been utilized for staining. Intracellular NO level was defined using flow cytometry technique.

**Flow cytometry analysis.** The inclusion of fluorescent dyes in BAL cells was immediately examined by using of Coulter Epics XL (Beckman Coulter, USA), equipped with argon laser, λex = 488 nm. Intensity of DAF fluorescence from cells pre-loaded with DAF-2DA was analyzed at FL1 filter characterized by λem = 525 ± 20 nm. Fluorescence intensity of EtBr staining cells was monitored at FL3 channel transmitting light band λem = 620 ± 15 nm. The measurement of cytometric parameters in each separate sample was processed in two parallels. A region (R1) was drawn to exclude cell debris based on forward (FSC) and side (SSC) scatter characteristics. All subsequent analysis was carried out on R1 gated cells. At least 10000 events were analyzed. Fluorescence signal alterations were defined according to fluorescence peak relatively to the marker, which set apart the autofluorescence peak indicating the unstained cells. The results were graphically presented using “FCS Express V3” (De Novo, USA).

**Statistical analysis.** All data analysis was done using statistical package (Statistica version 6.0). Normality was assessed using graphical plotting of distribution variables within groups. Levene’s median test was used to evaluate the homogeneity of variances. Data were not normally distributed. Therefore nonparametric testing was performed. A value of P < 0.05 was used as the cut-off point for significance. For two independent groups (Fig. 1A) Mann-Whitney P(U) test was defined. For multiple independent groups (Fig. 1B, 2–4) reliable changes were first assessed using Kruskal-Wallis ANOVA P(F) test and then group comparisons were carried out by post-hoc analysis using Newman-Keul’s P test, only when the Kruskal-Wallis ANOVA value was significant. All descriptive data are expressed as median and interquartile range given as [first - third quartiles]. Results in figures are shown in quartile box plots which indicated quartiles as the ends of the box; median as the black dot inside box; minimum and maximum as the whiskers extending from either end of the box. Since data are distributed nonnormally, associations between variables were examined by Spearman rank correlation test.

**Results and Discussion**

In order to gain insight into immune response in suggested guinea pig model of allergic asthma we determined plasma OVA specific IgG in two time-points after allergen hypersensitivity acquisition. The full development of the immune response takes up to 4 weeks from allergen injection. During this time, guinea pig OVA specific IgG, as well as IgE, are produced [17]. Here, guinea pig OVA specific IgG was defined at late stages of asthma development, namely 27 weeks later sensitization and after the last allergen provocation. Obtained results displayed that even 27 weeks later final immunization plasma OVA specific IgG level in OVA/0.9%NaCl group strongly differed from normal group (P(U)=0.0016) shown in Fig. 1A. Simultaneously present evidence was confirmed by findings that OVA specific IgG magnitude of OVA/0.9%NaCl animals (0.239[0.216-0.243] CU) largely overcome the threshold meaning of positive content of allergen specific IgG, which was equal to 0.076 CU, whereas normal group value (0.013[0.009-0.014] CU) did not exceed threshold. Marked increase in OVA specific IgG rate was still observed at the end of the experiment (33 weeks after sensitization) in plasma samples of OVA/0.9%NaCl animals compared to normal group (P(F) < 0.005; P = 0.0097; Fig. 1B). Moreover, repeated allergen aerosol challenge was accompanied with reliable changes of plasma OVA specific IgG relatively intact and sensitized animals (OVA/ OVA versus normal and OVA/0.9%NaCl respective-
Sensitization to allergen was associated with the growth of plasma IL-4 content (7.41 [3.45–14.24] pg/ml OVA/0.9%NaCl) in comparison with control (0.19 [0.62–1.30] pg/ml normal) as demonstrated in Fig. 2 (P (F) < 0.05; P = 0.019). Slightly reduced, but still significant IL-4 levels in plasma was present after OVA provocations (P (F) < 0.05; normal versus OVA/OVA: P = 0.016). Thus, multiple allergen inhalations resulted in narrowing of IL-4 interquartile range towards a trend to attenuation respectively sensitized animals (6.01 [5.86–6.45] pg/ml in OVA/OVA versus 7.41 [3.45–14.24] pg/ml in OVA/0.9%NaCl). IL-4 is a prime cytokine involved in allergic sensitization [18]. However, it has been noted that the cytokine profile in asthma varies with the number of allergen challenges [19].

Freshly collected BAL fluid of guinea pigs was assessed for cell survival by flow cytometric quantitation (Fig. 3). Cell death was significantly lower in OVA/0.9%NaCl as compared to normal group (1.39 [0.60–1.95] % versus 2.97 [2.58–3.18] %; P (F) < 0.05, P = 0.04), but were not statistically different from values of the OVA/OVA group (1.48 [1.15–4.03] %). Also no detectable differences were determined in percentage of dead cells in OVA/OVA compared to normal animals. In general, value of cellular viability in BAL fluid is considered to be high.

In addition to intrinsic shift in immune response, allergen exposures may also affect NO homeostasis. Therefore, we determined NO content in multi-cellular infiltrate recruited to the airways. Multiple allergen provocations of sensitized guinea pigs were associated with excessive level of intracellular NO generation compared to control and OVA sensitized animals (P (F) < 0.0001; OVA/OVA versus normal and OVA/0.9%NaCl respectively: P = 0.0002 and P = 0.0001; Fig. 4). Hence the amount of immune cells producing NO in BAL suspension reached a significant growth up to 33.50 [27.80–45.80] % in OVA/OVA after allergen challenge exposures. The obtained data agreed with the evidence that allergic inflammation is accompanied by exaggerated NO synthesis in the respiratory tract [6, 8, 10]. The increased levels of NO can be explained by NOS overexpression. NOS enzymes are composed of inducible NOS (iNOS/NOS2) and of constitutive isoforms: endothelial NOS (eNOS/NOS3) and neuronal NOS.
(nNOS/NOS1), which basically release moderate NO levels involved in the normal metabolic functions [20]. In patients with asthma, the exogenous stimuli originate from allergens and environmental pollutants, initiating transcripational activation of iNOS in the respiratory tract. Abnormally increased iNOS mRNA in protein and high NO output in patients with asthma are likely due to a continuous transcriptional regulation of the iNOS gene by IFNγ-JAK-STAT-1 protein activation [21].

In the airways of asthmatic patients [22] or in the rodent lung after allergen challenge [23], iNOS expression and/or enzymatic activity in lung tissue are increased. Hence, our findings concerned NO synthesis in inflammatory cells infiltrating respiratory tract after allergen challenges complement statement, that nitrosative stress in lungs and tracheal smooth muscles is deeply and permanently involved in exacerbation of Th2-mediated asthma. Besides, iNOS-derived NO is produced by genuine immune-system cells including monocytes and macrophages [24], eosinophils [25], neutrophils [26], dendritic cells [27].

The level of NO containing BAL cells was observed to be strongly reduced to 6.30[2.20–7.82]% in OVA/0.9%NaCl in contrast with normal guinea pigs ($P(F) < 0.0001; P = 0.0002$). There is no published data that have examined direct NO formation within cells of BAL suspension and its distribution in populations. An important question presents itself: how did the level of intracellular NO become extremely low after sensitization acquisition? The deficiency of NO synthesis in OVA/0.9%NaCl could be related to the cellular composition in BAL suspension and regulatory effects mediated by Th2 immune subset after allergen hypersensitivity acquisition. We identified alterations in cellularity of BAL fluid in depend of the experimental group as indicated in FACS density graphs (Fig. 5). This indicates redistribution of immune subpopulations in general pool of cells infiltrating respiratory tract at each next phase of asthma development (normal – immune response in balance, OVA/0.9%NaCl – sensitization to antigen, OVA/OVA – allergic inflammation induced by airway hyperresponsiveness). Moreover, all guinea pigs in OVA/0.9%NaCl group expressed distinctive cellular composition clearly differed from those in normal and OVA/OVA. Considering Th2-mediated regulatory networks, iNOS-suppressing activities of IL-4 and IL-10 are well established. IL-4 induced GATA-3 allows the stable commitment to the Th2 phenotype through promotion of Th2 cytokine production, such as IL-4, IL-5, IL-10 and IL-13 [28]. IL-10 produced by Th2 cells further suppresses Th1 development by inhibiting secretion of IFN-γ and IL-12. It may also directly inhibit the induction of iNOS [29], since IFN-γ is the most potent and prevailing inducer of iNOS in vivo [30].

Fig. 2. Guinea pig IL-4 plasma level under normal condition and OVA-induced asthma. A – content of IL-4 in guinea pig blood plasma, $P(F) < 0.05$ (Kruskal-Wallis ANOVA test). B – absorbance spectra of IL-4 plasma level in the wavelength range $\lambda = 380-600$ nm.
Fig. 3. Differentiation of viable and dead cells in bronchoalveolar lavage fluid of guinea pigs under normal condition and OVA-induced asthma. A – flow cytometry histograms of BAL cell viability. Histograms present a gate, which separates the cells labeled by EtBr (count – number of events, FL3LOG – relative fluorescence intensity of red spectrum in log scale). Purple and black curves match to the sample incubated with fluorescent dye and its unstained control (here and Fig. 4A). B – distribution of dead cells depending on size in BAL suspension. EtBr labeled cells are colored in red. C – quantitative analysis of cell death in guinea pig BAL fluid, \( P(F) < 0.05 \) (Kruskal–Wallis ANOVA)
Fig. 4. Intracellular NO content in BAL fluid of guinea pigs under normal condition and OVA-induced asthma. 
A – flow cytometry histograms of NO formation in the immune cells derived from guinea pig BAL fluid. Histograms present a gate, which separates the cells labeled by fluorescent dye (here and Fig. 5: FL1LOG – relative fluorescence intensity of green spectrum in log scale). B – cell types in BAL fluid based on the morphological flow cytometric parameters side scatter (SS) versus forward scatter (FS) are illustrated in color dot plots. DAF-2DA stained cells are colored in green here and in Fig. 5. C – levels of NO generating cells in infiltrate recruited to the lower respiratory tract: quantitative analysis, $P(F) < 0.05$ (Kruskal-Wallis ANOVA)
It is also important to consider BAL cellular NO homeostasis not in isolation but in the context of characteristic allergic asthma parameters. We have noticed that the substantial drop in percentage of NO containing BAL cells corresponded with reliably increased IL-4 plasma level in OVA/0.9%NaCl guinea pigs. However, in allergic inflammation induced by repeated antigen provocations significant enlargement of intracellular NO value was accompanied with significant IL-4 plasma level. High levels of NO generated by iNOS prevent overexpansion of Th1 cells [7]. NO markedly inhibits IL-2 production though impairment of IL-2 promoter induction, and increases the production of IL-4 by effector T cells [31]. Presented observations gives evidence that NO selectively inhibits the expansion of Th1 cells by a negative feedback mechanism and thus indirectly promotes formation of Th2 immune subset. No statistically significant correlation was observed between intracellular NO level in BAL cells and IL-4 plasma content. Although IL-4 was seen as the crucial cytokine in asthma, its production is transient [32]. It means that cumulatively, IL-4 is considered critical during the onset of the disease phenotype and may be overlapped in later stages of pathogenesis. These are in line with our findings concerning absence of correlation, since the IL-4R α chain is a common component of both the IL-4R and the IL-13R. However, the reverse is not true: T-cells respond to IL-4 but not to IL-13 [33].

Antigen-specific IgE production, with subsequent fixation of IgE to FcεRI receptors on mast cells and basophils, is central to the initiation and propagation of immediate hypersensitivity reactions. Association between extraordinarily high levels of IgE and either NOS activity or NO metabolites was found [34]. However, guinea asthma studies indicated a predominant role for IgG in the allergic response. Thus, although IgE antibodies, having a passive cutaneous anaphylactic activity, have been demonstrated [35], but in the experimental animals, such as the guinea pig, IgG1 appears to be the prevailing homocytotropic antibody. Importantly, we found that the level of NO formation in immune

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**Fig. 5.** NO formation depending on size (panel A) and granularity (panel B) in cell subpopulations derived from guinea pig BAL fluid
cells derived from guinea pig BAL fluid correlated in direct ratio with plasma OVA specific IgG content \((P = 0.0365; \ r = 0.7381)\).

To summarize, the identification of NO formation and hypersensitivity parameters under guinea pig model of acute asthma with multiple challenges revealed interrelation between characteristic immunologic parameters of the disease and NO levels formed immediately by inflammatory immune cells infiltrating airways. In the present experimental model of bronchial asthma significant association between plasma OVA specific IgG value and level of NO generating cells derived from guinea pig BAL fluid was detected. Here we demonstrated that NO formation in cellular infiltrate recruited to the lower respiratory tract is transformed from reduced to exaggerated level during Th2-dominated immune reaction in the presence of sensitization. In view of these findings, we infer that in asthma excessive NO generation is maintained not only due to increased iNOS protein expression and activity in lung tissue cells, but also due to NO synthesis in multicellular airway infiltrate recruited to trigger organ during allergic inflammation. Therefore high BAL levels of NO may reflect circulating nitrosative stress in respiratory tract when exacerbation in asthma is present. Taken together with the known Th1-mediated feedback regulation of iNOS, our results corroborate the viewpoint that NO derived from lower respiratory tract is a direct signal of the Th2-mediated pro-inflammatory cytokine mechanisms of central importance in the pathophysiology of allergic airway inflammation.

аллергического воспаления. Уровень генерации внутриклеточного NO значимо коррелировал с показателями аллергенспецифического IgG в плазме крови при OVA-индукции астмы. Приведенные данные свидетельствуют, что повышенный уровень внутриклеточного NO в BAL может отражать в целом нитрозативный стресс дыхательных путей при обострении аллергической астмы.

Ключевые слова: овальбумин-индукированная астма, сенсибилизация, оксид азота, аллергическое воспаление, бронхоальвеолярный лаваж.

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