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INDICATORS OF HUMORAL IMMUNITY UNDER CHEMICAL BURNS OF ESOPHAGUS IN RATS

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It is well known that the immune system has been actively involved in the regeneration and healing processes of post burn wounds. However, unanswered questions remain concerning the role of humoral immunity in the healing mechanisms and development of burn wound complications. We have developed an experimental model of chemical esophageal burn (CEB) which corresponds to esophageal burn in 1-8 years old children. We studied the features of humoral immunity upon CEB in rats. A decrease in IgG levels and an increase in levels of medium- and low- molecular circulating immune complexes (CIC) on the first day of esophageal burns were observed. On the 21st day of burn, we observed an increase in the IgG concentration and a tendency to accumulation of medium- and low-molecular CIC. The studied indicators can be used to differentiate CEB development and create a timeline of burn wounds.

K e y w o r d s: chemical esophageal burn in rats, IgG level, level of circulating immune complexes (CIC).

According to the World Health Organization reports, a steady increase in the number of chemical burns of the upper gastrointestinal tract is observed. It is associated with the increasing production of new chemicals, the continuing production of the concentrated acetic acid and a careless storage of chemicals in daily (domestic) life. According to statistics, the largest number of chemical poisoning (from 77.2 to 85.0%) occurs in children from 1 to 3 years old [1, 2].

The researches mainly focus on the pathogenesis of the development and treatment of esophageal burns [3, 4]. However, role of humoral immunity in the development of burn injury is little reported in literature and the published data are often contradictory. Thus, it was shown that the level of all immunoglobulin classes increased within two weeks after the chemical burn [5]. However, it was indicated in the work [6] that the level of M, G, A immunoglobulins decreased throughout the post-burn period. It should also be noted that the vast majority of researches of the immune system response to burn injury is dedicated to adults, while children immune system response has not been sufficiently studied [7]. Thus, the studies of peculiarity of children immune system response upon CEB of various natures and degrees are required.

The aim of this work was to analyze the distinct features of the immune response and their potential effect on the platelets functioning.

Materials and Methods

White wild rats (1-month, 90-110 g body weight) were used in experiments in compliance with provisions for the use of animals in biomedical experiments approved by the First Ukrainian National Congress on Bioethics (September 2001) and other international agreements and national legislation in this area [8]. Studied animals received a standard diet. Chemical burns in animals were experimentally modeled in the following way: alkaline esophageal burn was caused by 10% NaOH (1st degree burns) and 20% NaOH (2nd degree burns), acid esophageal burn was caused by 30% CCl_3COOH (2nd degree burns) [9].

The antibodies level in the blood of studied animals was determined by immunoenzymatic assay in 96-well microplates (Dynatech, Sweden) [9]. Contents of circulating immune complexes (CIC) in the serum were determined by precipitation: macromolecular CIC with 3% polyethylene glycol (PEG), medium molecular CIC with 4.5% PEG, low molecular CIC with 6% PEG (PEG 6000) [11]. The optical absorbance was read at $\lambda = 450$ nm by Titertek Multiskan spectrophotometer (Finland).

To obtain IgG fraction from the blood serum, 1 ml of serum was layered on a column with protein-A Sepharose (total column volume 5 ml). Nonspecifically bound proteins were washed with 0.05 M Tris-HCl buffer, pH 7.4 in a volume of tenfold of total column volume (50 ml). Elution was carried out using a glycine buffer (0.1 M glycine-HCl, pH 2.2). Samples containing protein were precipitated by ammonium sulfate solution (final concentration 50%) and were left at 4 °C overnight. The precipitate was centrifuged at 3000 rpm/min for 30 min. The supernatant was withdrawn and the precipitate was dissolved in 1 ml of 0.05 M Na-phosphate buffer, pH 7.4 [12]. To remove ammonium residues, the solution of antibodies was applied to column G 25 equilibrated with 0.05 M Na-phosphate buffer, pH 7.4 (total column volume 50 ml). Samples containing protein were concentrated, then absorbance was measured and the antibody concentration was calculated. The obtained samples were stored at - 20 °C. The IgG fractions from serum were isolated on the 15th day of the experiment.

The release of platelet protein in the studied animals was analyzed by disc-electrophoresis with Laemmli system [13], followed by Western blotting [14]. Platelets were incubated with the IgG fractions (final concentration 1 mg/ml) for 30 min at 37 °C and the cells were precipitated by centrifugation at 300 rpm, 10 min. Platelet incubation medium was used as a sample for analysis. Quantitative analysis of electrophoreses and immunoblots were performed using the software TotalLab 2.01.

Statistical data analysis was performed using the software OriginLab 8.0. and TotalLab 2.01. The values were considered significant at $P < 0.05$; value alterations at $P > 0.05$ were considered as a tendency.

Results and Discussion

The degree of pathological reactions of various organs depends on the severity of burn injuries, timely and sufficient treatment or the age of the patient [15, 16]. In our experiment we used a model that sufficiently imitates the chemical burn of the esophagus in children 1-8 years old [9] and allow finding factors that characterize different degree of injury as well as peculiarities of mechanism of child's immune system response in case of CEB. Mortality from burns depends on the degree, severity and stage of injury [17].

The obtained data indicate that CEB alters the functioning of parts of humoral immunity that agreed to the published data [7]. A significant decrease in IgG (the most specific humoral effector) level in serum on the 1st day of CEB was observed (Table 1).

It was found that the level of antibodies decreased more in the animals with 2nd degree alkaline esophageal burn and 2nd degree acid esophageal burn than in the animals with 1st degree acid esophageal burn (Table 1) by 12, 84 and 48% respectively in comparison with control. These alterations indicate the probable correlation between the degree of chemical burn and the level of serum antibodies.

CIC level in blood is one of the indicators of the immune system status. The size of immune complexes plays an important role in the immune complex formation, owing to small and middle sized immune complexes being the most pathogenic and capable of activating the complement system. Immune complexes level is an indicative feature in case of burn although problems associated with appropriateness of identification of CIC upon CEB have not been resolved yet.

It was shown that CEB caused alterations of CIC level on the 1st day of the burn injury (Table 2).

We observed the tendency to elevation of the level of high-molecular CIC in animals with CEB in comparison with control. The obtained values exceeded control by 10% in animals with the 1st degree alkaline esophageal burn, by 20% in animals with 2nd degree alkaline esophageal burn, and by 30% in animals with 2nd degree acid esophageal burn.

The level of medium-molecular CIC in animals with the 2nd degree alkaline esophageal burn was the highest and exceeded that in control group by 35% that indicated that inflammatory processes devel-

Table 1. IgG level in rats blood serum on the first day post experimental chemical esophageal burn ($M \pm m$, $n = 10$)

Animal group	IgG level (arb. unit)
Control	0.46 ± 0.02
Alkaline esophageal burn, 1st degree	0.41 ± 0.01*
Alkaline esophageal burn, 2nd degree	0.25 ± 0.01*
Acid esophageal burn, 2nd degree	0.31 ± 0.03*

Here and in Table 2–4 * $P < 0,05$ with respect to control.

Table 2. Level of circulating immune complexes (CIC) in rat blood serum on 1st day post experimental chemical esophageal burns (M ± m, n = 10)

Animal group	High-molecular CIC (arb.unit)	Medium-molecular CIC (arb.unit)	Low-molecular CIC (arb.unit)
Control	0.10 ± 0.001	0.14 ± 0.001	0.12 ± 0.001
Alkaline esophageal burn, 1st degree	0.11 ± 0.025	0.17 ± 0.004*	0.15 ± 0.016*
Alkaline esophageal burn, 2nd degree	0.12 ± 0.017*	0.19 ± 0.015*	0.18± 0.043*
Acid esophageal burn, 2nd degree	0.13 ± 0.015*	0.18 ± 0.02*	0.17 ± 0.014*

oped. We found that CIC level in animals with the 1st degree alkaline esophageal burn and with 2nd degree acid esophageal burn increased by 21 and 28%, respectively.

It was observed that the level of low-molecular CIC also tended to increase in animals with CEB: in animals with 1st degree alkaline esophageal burn exceeded control by 25%, in animals with 2nd degree alkaline esophageal burn by 50% and in animals with 2nd degree acid esophageal burn by 41%.

An increase in CIC level on the 1st day of burn injury in all animal groups with CEB draws attention. CIC level in rats with the 2nd degree alkaline esophageal burn and 2nd degree acid esophageal burn was higher than that in animals with 1st degree alkaline esophageal burn.

Thus, it was noticed that a decrease in IgG level on the 1st day of the CEB development was accompanied with significant changes in the molecular composition of immune complexes. It was primarily evident from the increased concentration of the most toxigenic medium- and low-molecular CIC.

As it can be seen in Table 3, a significant increase in IgG level in blood serum occurred on the

Table 3. IgG level in serum of rats with experimental CEB on the 21st day post burn injury (M ± m, n = 10)

Animal group	IgG level (arb. unit)
Control	0.46 ± 0.02
Alkaline esophageal burn, 1st degree	0.51 ± 0.03
Alkaline esophageal burn, 2nd degree	0.61 ± 0.02*
Acid esophageal burn, 2nd degree	0.66 ± 0.03*

21st day post burn injury in rats with experimental CEB.

Assessing IgG levels in the experimental animals we found that antibody levels in animals with the 2nd degree alkaline esophageal burn and 2nd degree acid esophageal burn exceeded that in the control group by 32 and 43%, respectively.

Investigating the level of low-molecular CIC in rats with CEB on the 21st day post burn injury, we observed a tendency to a slight increase in CIC level in comparison with the control.

The level of medium- and low-molecular CIC was the highest in animals with induced the 2nd degree alkaline esophageal burn and increased by 28 and 25%. The level of medium and low-molecular CIC tended to increase in animals with induced the 1st degree alkaline esophageal burn and 2nd degree acid esophageal burn. It was shown that the level of these CIC depended on the nature of chemical burns and was the highest for the 2nd degree alkaline esophageal burn and 2nd degree acid esophageal burn. Thus, the prolonged accumulation of CIC may be the main factor of tissue damage and may be associated with the development of immune complex pathological processes, particularly on the 21st day post burn injury [18].

The peculiarity of the immune system condition of animals with CEB on the 21st day is determined by activation of the majority factors functioning. Analysis of the clinical data [17] allowed us to suggest that the activation of immunological functions could not be regarded as evidence of normalization or recovery. This activation might be a manifestation of the high lability of the majority of the studied features and might be caused by the immaturity of organism's physiological systems.

Platelets play an important role in the development of the immune response in patients with burns. They release inflammatory mediators, express proinflammatory molecules, interact with leukocytes and

Table 4. Circulating immune complexes (CIC) levels in serum of rats with experimental chemical esophageal burns on the 21st day post burn injury (M ± m, n = 10)

Animal group	High-molecular CIC (arb. unit)	Medium-molecular CIC (arb. unit)	Low-molecular CIC (arb. unit)
Control	0.10 ± 0.001	0.14 ± 0.001	0.12 ± 0.001
Alkaline esophageal burn 1st degree	0.11 ± 0.025	0.17 ± 0.004	0.14 ± 0.016
Alkaline esophageal burn 2nd degree	0.10 ± 0.017*	0.18 ± 0.015*	0.15 ± 0.043*
Acid esophageal burn 2nd degree	0.11 ± 0.015*	0.16 ± 0.02*	0.15 ± 0.014*

endothelial cells, participating, this way, in the induction of both acute and chronic processes [19]. IgG and immune complexes are capable of activating the cells involved in the regulation of homeostasis (including platelets) [20]. The platelets are a source of plasminogen activator inhibitor type 1 (PAI-1). The concentration of PAI-1 in platelets exceeds plasma pool fourfold. It has been shown that approximately 70% of platelet PAI-1 is contained in the α-granules in active form which is biologically functional and capable of interacting with tissue plasminogen activator (tPA) [21]. Inhibitor is an important component of the fibrinolysis system, since it is the main regulator of the activity of tPA and urokinase [22]. Besides, PAI-1 is a marker of various pathological conditions. Thus, inhibitor concentration in plasma in case of sepsis increases approximately 100 times [20, 23]. It is believed that inhibition of PAI-1 may be a useful therapeutic strategy for reducing both inflammatory and prothrombotic processes [24].

It was shown that antibodies of control rats induced the release of 11 protein components from platelets at that the component with a molecular mass around 250 kDa was secreted in the largest amount (Fig. 1).

Antibodies of rats with CEB induced the release of protein from platelets, which exceeded that in the control sample by about 60%. It was shown that the content of components with molecular mass around 400, 350 and 250 kDa increased most significantly, by 121, 300, and 53% respectively. The release of component with molecular mass around 50 kDa also significantly increased, by 471%.

IgG in rats with alkaline esophageal burn induced increased release of the protein with molecular mass around 70 kD and low molecular components compared to those of control rats and IgG of rats with the 2nd degree acid esophageal burn. Protein with molecular mass 70 kDa was secreted 174% more, while the component with molecular mass 20 kDa was secreted by 405% more. The highest level of secretion of component with molecular mass 70 kDa was observed under the influence of antibodies of rats with the 2nd degree alkaline esophageal burn. This component considering its molecular mass can correspond to a heat shock protein (hsp 70) [25].

The effect of IgG of experimental animals on the secretion of plasminogen activator inhibitor type 1 (PAI-1) was studied using Western blot analysis.

Seven forms of the inhibitor were revealed on immunoblot in a control sample (Fig. 2). Antibodies produced in the body due to a burn injury led

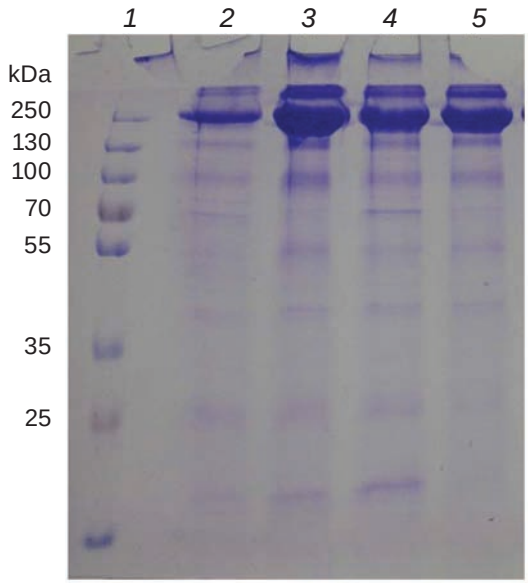

Fig. 1. Electrophoreses of media of incubation of rat platelets with IgG: 1 – markers; 2 – control rats; 3 – rats with the 1st degree alkaline esophageal burn (caused by 10% NaOH); 4 – rats with 2nd degree alkaline esophageal burn (caused by 20% NaOH); 5 – rats with 2nd degree acid esophageal burn (caused by 30% CCl_3COOH)

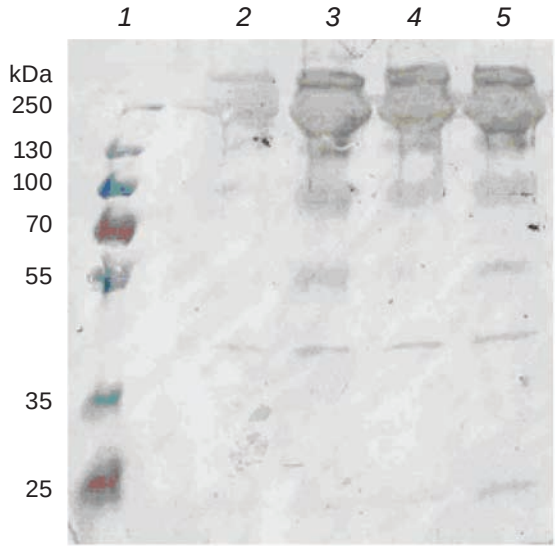

Fig. 2. Immunoblot of media of incubation of rat platelets with IgG: 1 – markers; 2 – control rats; 3 – rats with the 1st degree alkaline esophageal burn (caused by 10% NaOH); 4 – rats with 2nd degree alkaline esophageal burn caused by 20% NaOH; 5 – rats with 2nd degree acid esophageal burn (caused by 30% CCl_3COOH)

to increased secretion of PAI-1 by almost 289% compared to control. Inhibitor complexes with molecular masses around 400, 350, 250 and 140 kDa were released more intensively. The presence of an additional band (50 kDa), which may correspond to the free form of PAI-1, was also observed. The band around 100 kDa was detected in each sample. This band may correspond to a complex of PAI-1 with urokinase.

Antibodies of rats with CEB also induced an increase in fragmentation of this inhibitor. Thus, the intensive formation of the fragment (20 kDa) was observed under the influence of IgG produced during experimental 2nd degree acid esophageal burn. The antibodies produced as a result of the effect of both agents led to increased formation of PAI-1 fragment (40 kDa).

The most intensive alterations in the profile of platelet secretion were observed in the area of macromolecular complexes of PAI-1 and component 70 kDa which was only discovered by the electrophoresis.

Thus, CEB was accompanied by significant changes in humoral immunity. On the 1st day of the CEB development in rats we observed a decrease in IgG level and significant alterations in the molecular composition of immune complexes that was primarily evident from increased concentrations of the most toxigenic medium- and low-molecular CIC.

On the 21st day post burn injury a significant increase in the level of IgG occurred in serum of rats with experimental CEB. In animals with the 1st degree alkaline esophageal burn or 2nd degree acid esophageal burn the level of medium and low-molecular CIC tended to increase. It was shown that the CIC level depended on the nature of chemical burns and was the highest in case of 2nd degree alkaline esophageal burn.

IgG in rats with CEB increased the release of platelet proteins by 60% relative to control. IgG produced during 2nd degree alkaline esophageal burn caused higher secretion of the protein 70 kDa and low molecular components in comparison with IgG formed during the development of acid esophageal burn. IgG in both animal groups increased almost fourfold the release of PAI-1 compared to control.

ПОКАЗНИКИ ГУМОРАЛЬНОЇ ЛАНКИ ІМУНІТЕТУ ЗА ХІМІЧНОГО ОПІКУ СТРАВОХОДУ В ЩУРІВ

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Загальновідомо, що імунна система бере активну участь у процесах регенерації та загоєння післяопікових ран. Проте залишаються відкритими питання стосовно ролі гуморальної ланки імунітету в механізмах загоєння та розвитку післяопікових ускладнень. В експерименті на щурах відтворена модель хімічних опіків стравоходу (ХОС), що відповідає опікам стравоходу в дітей віком 1-8 років. Дослідженнями показників гуморального імунітету в умовах моделювання ХОС показано зниження вмісту IgG та підвищення концентрації середньо- та низькомолекулярних циркулюючих імунних комплексів (ЦІК). На 21-шу добу концентрація IgG збільшилась і спостерігалась тенденція до накопичення середньо- та низькомолекулярних ЦІК. Очищена фракція IgG сироватки крові щурів з ХОС спричинювала посилення секреції протеїнів тромбоцитів. Досліджені показники можуть бути застосовані для диференціації розвитку ХОС та хронізації опікової травми.

Ключові слова: хімічний опік стравоходу, рівень IgG, рівень циркулюючих імунних комплексів.

ПОКАЗАТЕЛИ ГУМОРАЛЬНОГО ЗВЕНА ИММУНИТЕТА ПРИ МОДЕЛИРОВАНИИ ХИМИЧЕСКОГО ОЖОГА ПИЩЕВОДА У КРЫС

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Общеизвестно, что иммунная система принимает активное участие в процессах регенерации и заживления послеожоговых ран. Однако остаются открытыми вопросы о роли гуморального звена иммунитета в механизмах заживления и развития послеожоговых осложнений. В эксперименте на крысах воспроизведена модель химических ожогов пищевода (ХОП), адекватная ожогу пищевода у детей 1–8 лет. Исследованиями показателей гуморального иммунитета в условиях моделирования ХОП показано снижение уровня IgG и повышение концентрации средне- и низкомолекулярных циркулирующих иммунных комплексов (ЦИК). На 21-е сутки происходит повышение уровня IgG и наблюдается тенденция накопления средне- и низкомолекулярных ЦИК. Очищенная фракция IgG сыворотки крови крыс с ХОП вызывала усиление секреции тромбоцитарных протеинов. Исследованные показатели могут быть использованы для дифференциации развития ХОП и хронизации ожоговой травмы.

Ключевые слова: химический ожог пищевода, уровень IgG, уровень циркулирующих иммунных комплексов.

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