UDC 616.931:579.871.1:612.017.4: 57.033

DETERMINATION OF REGIMES FOR DIPHTHERIA EXOTOXIN MODIFICATION BY CHEMICAL AND PHYSICOCHEMICAL METHODS

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At the present stage of immunobiological industry development unsafeness of many vaccine preparations is associated with the presence of harmful substances, which are used for detoxification and subsequent storage of the substrate and with the presence of ballast proteins, which can cause formation of nonspecific antibodies and reinforce reactogenicity of preparations and their sensitizing properties during immunization.

Nowadays toxoids are obtained by the addition of 0.6-1.5 per cent of formalin (in terms of formaldehyde content 40%) to the toxigenic microbes culture filtrates [1-3]. Formaldehyde is well known cross-linking agent that can inactivate, stabilize or immobilize protein molecules. Depending on their sequence, peptides undergo a great diversity of chemical modifications after formaldehyde treatment: the formation of methylol groups, Schiff-bases, and methylene bridges. The formation of methylol and Schiff-bases is reversible, and therefore these compounds are generally hard to detect. The most important modification of peptides (and proteins) induced by formaldehyde is the formation of stable methylene bridges. It is shown that only primary amino and thiol groups primarily react with formaldehyde and form cross-links in a second step with several other amino acid residues, i.e. with arginine, asparagine, glutamine, histidine, tryptophan, and tyrosine residues. In contrast to these cross-link reactions, no methylene bridges were formed between two primary amino groups [4].

Thus, under certain conditions formaldehyde treatment of bacterial toxins results in violations of the toxic protein spatial configuration at the expense of cross-links between individual plots of toxin polypeptide chain or between its individual subunits, that prevents the dissociation of the toxin molecule into two fragments and the toxigenic fragment A liberation [1 - 4]. Thereby, the inactivation (neutralization) of bacterial toxins is achieved by modification of their native structure.

The question regarding the possibility of diphtheria exotoxin detoxifying with chemicals less toxic than formaldehyde has been insufficiently explored. Crystalline vitamin C was proposed as an alternative modifier by some researchers (C.W Jungblut, R.L. Zwemer). Ambiguous results were obtained during the simultaneous introduction of exotoxin with ascorbic acid and toxin pretreated with the modifier to laboratory animals. Some publications defined a high protective effect of vitamin C the others showed opposite results [5, 6]. This fact had not been researched later on.

Considered data don't provide an answer to the question about the role of toxoid components in the humoral immunity formation and don't assign the limits of its purification, which does not reduce immunogenic properties of the vaccine.

The aim of our work was to study the possibility of replacement of harmful modifiers by safer chemicals that contain  functional group (amino sugars), as well as by some representatives of organic acids, that contain carboxyl group  . Ultrasound (US) (ultrasonic device TR 3468-001-42369179-03) was used for the possible acceleration of diphtheria toxin detoxication, because ultrasonic vibrations can accelerate the chemical processes and result in new reaction products formation due to cavitation.

Materials and methods

Flocculation test was used for determination of diphtheria toxin or toxoid specific (antigenic) activity of [7]. Standard preparation of diphtheria antitoxin for flocculation test was used (OCO 42-28-249-10 П, from Tarasevich State Institute of Standardization and Control of Biomedical Preparations, Russia). Standard antitoxin was ex tempore diluted in isotonic sodium chloride to a final concentration of 200 Lf per 1 ml.

Diluted standard antitoxin for flocculation test in appropriate concentrations and 2 ml of toxin or toxoid that was investigated were placed together in tubes and were thoroughly stirred. The mixtures were incubated in a water bath at 45-50 ^{0}C. The mixture flocculating first was that which contained the most nearly equivalent quantities of toxin and antitoxin [7]. The Limes flocculation (Lf) of diphtheria toxin was calculated by the formula:

$$A = \frac{V \times 200}{2},$$

where A is the Limes flocculation (Lf);

V is equivalent volume of diphtheria antitoxin for flocculation test;

200 is recalculation in terms of standard antitoxin dilution (200 Lf/ml);

2 is recalculation in terms of toxin (toxoid) volume in the sample.

The toxicity of diphtheria toxin and modified preparations was determined by intradermal method (dermonecrotic test) [7]. Laboratory animals (guinea-pigs with weight 350-400 g) were shaved on each side and 0.2 ml of the modified toxin preparations were injected intradermally into depilated guinea-pig skin. The animals were observed during 72 hours. The results were described in

following manner: 0 - absence of characteristic signs of epidermis damage, I - hyperemia, II - the presence of infiltration, III - necrosis. The preparation was identified as nontoxic if it didn't cause the local reactions, or caused only hyperemia, which disappeared within 72 hours [7].

Test for determination of diphtheria toxoid safety [7]. The modified toxin (potential toxoid) preparations were injected subcutaneously (2.5 ml into each side, total 5.0 ml) to laboratory animals (guinea-pigs with weight 350-400 g). The animals were observed during 45 days. The preparation was considered safe if laboratory animals put on weight during the observation time they didn't have paralysis, paresis, and local reactions (small indurations were allowed in the site of injection for 30 days or more) [7].

Statistical data processing was carried out in accordance with the rules of ordinary and alternative variation statistics [8, 11]. The calculations were done by the instrumentality of software Statistika-6, Microsoft Office Excel 2003.

Results and discussion

40% formalin was used as modifier number 1, modifiers №№ 2-3 were presented by organic acids and modifier number 4 was presented by amino sugar.

In the first stage of our research the volume content (0.3-0.6%) of investigated modifiers in native diphtheria toxin (DT) was estimated and the activity of obtained diphtheria toxin modifications was studied in the flocculation test (Table 1).

The data (Table 1) show that modifiers volume content decreasing in 2 times didn't have significant influence on toxin derived modifications activity. Adding the two components (amino sugar and formalin) simultaneously or after a certain period of time led to 3.5-5.0 fold increase of flocculation time ($p \leq 0,01$) in comparison with the samples containing only one modifier.

Processing of the diphtheria toxin modifications with ultrasound, in all trials except sample number 19, led to 1.5-3.7 fold increase of flocculation time ($p \leq 0,05$) in comparison with appropriate modifications untreated with ultrasound. The processing of the sample number 19 with above mentioned physical factor did not affect the flocculation time, however, the activity of obtained DT modification increased by 20 Lf.

Table 1.- Activity of diphtheria toxin modifications in the flocculation test

№	Diphtheria toxin derived modifications	The Limes flocculation (Lf), (M±m)	The time of flocculation, (min.)
1	Native diphtheria toxin (DT)	(60±5)	15
2	DT + (0,3)% modifier №1	(80±5)	20
3	DT + (0,3)% modifier №2	(60±5)	17
4	DT + (0,3)% modifier №3	(60±5)	17
5	DT + (0,3)% modifier №4	(60±5)	15
6	DT + (0,6)% modifier №1	(70±5)	15
7	DT + (0,6)% modifier №2	(60±5)	18
8	DT + (0,6)% modifier №3	(60±5)	18
9	DT + (0,6)% modifier №4	(70±5)	18
10	DT + (0,6)% modifier №4 + (0,6)% modifier №1 simultaneously	(60±5)	70
11	DT + (0,6)% modifier №4 + (0,6)% modifier №1 after 40 days	(60±5)	75
12	DT + (0,3)% modifier №1 + US	(80±5)	55
13	DT + (0,3)% modifier №2+ US	(60±5)	25
14	DT + (0,3)% modifier №3+ US	(60±5)	45
15	DT + (0,3)% modifier №4+ US	(60±5)	55
16	DT + (0,6)% modifier №1 + US	(70±5)	50
17	DT + (0,6)% modifier №2 + US	(70±5)	55
18	DT + (0,6)% modifier №3 + US	(80±5)	31
19	DT + (0,6)% modifier №4 + US	(90±5)	17
20	DT + (0,6)% modifier №4 + (0,6)% modifier №1 simultaneously + US	(70±5)	50

In the second phase of this research toxicity and safety of received diphtheria toxin modifications were investigated. It was experimentally determined that diphtheria toxin modifications obtained by the instrumentality of modifier number 1 were safe and non-toxic (Table 2).

Table 2.- Toxicity and safety of diphtheria toxin modifications

№	Diphtheria toxin derived modifications	Toxicity (24 hours / 72 hours)	Safety
1	Native diphtheria toxin (DT)	III/III	-
2	DT + (0,3)% modifier №1	I/0	+

3	DT + (0,3)% modifier №2	III/III	-
4	DT + (0,3)% modifier №3	III/III	-
5	DT + (0,3)% modifier №4	III/III	-
6	DT + (0,6)% modifier №1	I/0	+
7	DT + (0,6)% modifier №2	III/III	-
8	DT + (0,6)% modifier №3	III/III	-
9	DT + (0,6)% modifier №4	III/III	-
10	DT + (0,6)% modifier №4 + (0,6)% modifier №1 simultaneously	II/I	+
11	DT + (0,6)% modifier №4 + (0,6)% modifier №1 after 40 days	II/I	+
12	DT + (0,3)% modifier №1 + US	I/0	+
13	DT + (0,3)% modifier №2+ US	III/III	-
14	DT + (0,3)% modifier №3+ US	III/III	-
15	DT + (0,3)% modifier №4+ US	III/III	-
16	DT + (0,6)% modifier №1 + US	0/0	+
17	DT + (0,6)% modifier №2 + US	III/III	-
18	DT + (0,6)% modifier №3 + US	III/III	-
19	DT + (0,6)% modifier №4 + US	III/III	-
20	DT + (0,6)% modifier №4 + (0,6)% modifier №1 simultaneously + US	II/I	+

Notes:1. 0 – absence of epidermal reactions, I - hyperemia, II – infiltration, III – necrosis; 2. «+» - a preparation is safe (animals are alive and they put on weight); 3. «-» - a preparation is unsafe (animals died).

It should be noted that epidermal reaction in guinea-pigs was completely absent after intradermal introduction of the sample containing high volume of modifier number 1 and treated with ultrasound, while hyperemia was expressed in animals during the first observation day after introduction of the similar derivate untreated with ultrasound.

Comparative study of immunogenicity and protective properties of toxoids-candidates obtained by the instrumentality of some physical and chemical factors are planned in further experiments.

Conclusions:

1. The possibility of diphtheria toxoid obtaining using chemical (amino sugars, organic acids) and physicochemical (amino sugars, organic acids, ultrasound, temperature) factors was studied.

2. It was determined that modifiers (including formaldehyde) volume content decreasing didn't have significant influence on diphtheria toxin derived modifications specific activity.

3. Adding the two components (amino sugar and formalin) simultaneously or after a certain period of time (40 days) led to 3,5-5,0 fold increase of flocculation time ($p \leq 0,01$) in comparison with the samples containing only one modifier.

4. Processing of the diphtheria toxin modifications with ultrasound, in most samples led to 1,5-3,7 fold increase of flocculation time ($p \leq 0,05$) in comparison with appropriate modifications untreated with ultrasound.

5. The samples containing 0,6% of modifiers number 3 and number 4, after processing with ultrasound increased its specific activity, on average, by 20 ± 5 Lf in comparison with the samples untreated with ultrasound.

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UDC 616.931:579.871.1:612.017.4: 57.033

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The possibility of diphtheria toxoid obtaining using chemical (amino sugars, organic acids) and physicochemical (amino sugars, organic acids, ultrasound, temperature) factors was studied. It was established that modifiers (including formaldehyde) volume content decreasing didn't have significant influence on diphtheria toxin derived modifications specific activity. It was experimentally determined that diphtheria toxin modifications obtained by the instrumentality of modifier number 1 with or without the physical factor processing were safe and non-toxic.

Key words: diphtheria toxin, toxoid, chemical factors, physicochemical factors.

УДК 616.931:579.871.1:612.017.4: 57.033

ВИЗНАЧЕННЯ РЕЖИМІВ МОДИФІКАЦІЙ ДИФТЕРІЙНОГО ЕКЗОТОКСИНУ ФІЗИКО-ХІМІЧНИМИ МЕТОДАМИ

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Вивчено можливість одержання дифтерійного анатоксину за допомогою хімічних (аміноцукри, органічні кислоти) та фізико-хімічних (аміноцукри, органічні кислоти, ультразвук, температура) чинників. Визначено, що зменшення об'ємної долі модифікантів, у тому числі формаліну, в два рази, суттєво не впливає на специфічну активність отриманих дериватів дифтерійного токсину. Експериментально встановлено, що безпечними і нетоксичними були похідні дифтерійного токсину, які були отримані за допомогою модифіканта №1, як з обробкою, так і без обробки фізичним чинником.

Ключові слова: дифтерійний токсин, анатоксин, хімічні чинники, фізикохімічні чинники.

УДК 616.931:579.871.1:612.017.4: 57.033

ОПРЕДЕЛЕНИЕ РЕЖИМОВ МОДИФИКАЦИЙ ДИФТЕРИЙНОГО ЕКЗОТОКСИНА ФИЗИКО-ХИМИЧЕСКИМИ МЕТОДАМИ

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Изучена возможность получения дифтерийного анатоксина с помощью химических (аминосахара, органические кислоты) и физико-химических методов (аминосахара, органические кислоты, ультразвук, температура) факторов. Определено, что снижение объемной доли модификантов, в том числе и формалина, в два раза, существенно не влияет на специфическую активность дериватов дифтерийного токсина. Экспериментально установлено, что безопасными и нетоксичными были производные дифтерийного токсина, полученные с помощью модификанта №1, как с обработкой, так и без обработки ультразвуком.

Ключевые слова: дифтерийный токсин, анатоксин, химические факторы, физико-химические факторы.