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DYNAMICS OF IMMUNOLOGICAL REACTIVITY INDICES IN PATIENTS WITH LARYNGAL PAPILLOMATOSIS AT DIFFERENT PERIODS AFTER COMPLEX TREATMENT WITH ANTIVIRUS QUDRIVALENT VACCINE "GARDASIL"

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Ключові слова: *папіломатоз гортані, імунологічна реактивність, протирецидивна терапія, квадривалентна противірусна вакцина*
Ключевые слова: *папилломатоз гортани, иммунологическая реактивность, противорецидивирующая терапия, квадривалентная противовирусная вакцина*

Abstract. Dynamics of immunological reactivity indices in patients with laryngal papillomatosis at different periods after complex treatment with antivirus qudrivalent vaccine "Gardasil". Zabolotny D.I., Sambur M.B., Savchenko T.D., Krivokhatskaya L.D., Zayets T.A., Tymchenko M.D. *The aim of the study is to determine disorders of immune homeostasis in patients with laryngeal papillomatosis at different periods after complex treatment with the inclusion of the "Gardasil" quadrivalent vaccine in the adjuvant therapy. Studies of the immunity state were conducted in 26 patients with laryngeal papillomatosis (LP) before, 2-12 and 13-29 months after complex treatment, consisting of surgical removal of papillomas and anti-relapse therapy, including inhalation of Laferobion, taking a vitamin-mineral complex and a course of vaccination with antiviral "Gardasil" quadrivalent vaccine according to*

which the second and third injections were administered in 2 and 6 months after the first. Significant disorders of cellular and humoral immunity indices in patients with LP were revealed in the form of multidirectional changes in the functional activity of natural killer cells, a decrease in the total number of T lymphocytes, mainly due to $CD4^+$ subpopulation, increase in circulating immune complexes level in blood serum and concentration of secretory IgA in the secretion of the oropharynx. Disbalance in the patient's cytokine system resulted in increased γ-IFN and TNF-α serum levels, an increase in spontaneous production of interferon and inhibition of stimulated production of λ - and γ-interferon by blood cells in vitro. Complex treatment with the use of the "Gardasil" quadrivalent antiviral vaccine in the postoperative period contributed to the normalization of most immunological reactivity indices that were altered in patients. The results indicate the prospects of using the antiviral vaccine as part of adjuvant antirelapse therapy after surgical treatment of LP patients and indicate the advisability of long-term clinical and immunological monitoring at different periods after vaccination.

Реферат. Динамика показаталей иммунологической реактивности больных папилломатозом гортани в разные сроки после комплексного лечения с применением противовирусной квадривалентной вакцины «Гардасил». Заболотный Д.І., Самбур М.Б., Савченко Т.Д., Кривохатская Л.Д., Заец Т.А., Тимченко М.Д. *Цель работы - определение нарушений иммунного гомеостаза у больных паппиломатозом гортани в разные сроки после комплексного лечения с включением в состав адъювантной терапии квадривалентной вакцины «Гардасил». Исследования состояния иммунитета были проведены у 26 больных папилломатозом гортани (ПГ) до, через 2-12 и 13-29 месяцев после комплексного лечения, состоявшего из хирургического удаления папиллом и противорецидивирующей терапии, включавшей ингалации лаферобиона, прием витаминно-минерального комплекса и курс вакцинации противовирусной квадривалентной вакциной «Гардасил», который проводился по схеме, по которой вторая и третья инъекции вводилась через 2 и 6 месяцев после первой. Существенные нарушения показателей клеточного и гуморального иммунитета у больных ПГ были выявлены в виде разнонаправленных изменений функциональной активности естественных клеток-киллеров, уменьшения общего количества Т-лимфоцитов, в основном за счет $CD4^+$ - субпопуляции, повышения уровня циркулирующих иммунных комплексов в сыворотке крови и концентрации секреторного IgA в секрете ротоглотки. Дисбаланс в системе цитокинов больных выразился в повышении сывороточных уровней γ-ИФН и TNF-α, усилении спонтанной продукции интерферона и угнетении стимулированной продукции λ - и γ-интерферонов клетками крови in vitro. Комплексное лечение с использованием в послеоперационном периоде квадривалентной противовирусной вакцины «Гардасил» способствовало нормализации большинства измененных у больных показателей иммунологической реактивности. Полученные результаты свидетельствуют о перспективности использования противовирусной вакцины в составе адъювантной противорецидивирующей терапии после хирургического лечения у больных с ПГ и указывают на целесообразность проведения длительного клинического и иммунологического мониторинга в разные сроки после вакцинации.*

The disease of laryngeal papillomatosis (LP) is characterized by a progressive, recurrent course, capable of malignancy, which negatively affects the quality of life of patients, has serious social and financial consequences. Today it is considered proven that the main etiological factor of its development is the human papilloma virus (HPV) [10, 11, 13]. Respiratory papillomatosis is most often associated with viruses of types 6 and 11, which are classified as low carcinogenic risk, less often – 16, 18 types of high carcinogenic risk, which are considered to determine the most aggressive course of the disease. [10]. The frequency of malignancy of respiratory papillomatosis according to various authors is 2-3%. Thus, early diagnosis of the disease, increasing the effectiveness of its treatment are important factors in the prevention of laryngeal cancer.

The main method of LP treatment is surgical removal of the papilloma. However, due to its ability to spread to the upper respiratory tract and frequent recurrence, treatment of this pathology is still a problem and requires a comprehensive approach with the use of antirelapse adjuvant therapy in the postoperative period. Modern approaches to the adjuvant treatment of LP include immunomodulatory, antiviral therapy, the use of cytostatics and others [14]. In recent years, there are studies that indicate the inclusion of prophylactic vaccines against HPV in the complex therapy [17]. To date, three vaccines have been registered that provide protection against several types of human papillomavirus. The first generation vaccines include Cervarix® (GlaxoSmithKline), Gardasil-4® (Merck & Co., Inc., Whitehouse Station, NJ, USA). Cervarix is a bivalent recombinant vaccine that contains virus-like parts of HPV types 16 and 18, which cause most cases of oral and laryngeal cancer from the upper respiratory tract.

Gardasil-4 is a quadrivalent non-infectious recombinant HPV vaccine containing virus-like parts characteristic of HPV types 6, 11, 16, and 18. Vaccines do not contain viral genetic material but consist of the main capsid proteins L1, which induce

the development of humoral immune responses, they genetically engineered, not capable of reproduction and are not infectious [16].

In 2014, the US Food and Drug Administration (FDA) licensed the second-generation Gardasil-9 HPV vaccine, which in addition to four types (HPV 6, 11, 16, 18) contains antigens. types of HPV with high oncogenic risk (HPV 31, 33, 45, 52 and 58) and can prevent up to 93% of cases of cancer associated with HPV [12]. Studies have shown that Gardasil-9 vaccine is safe, generally well tolerated, and its high efficacy (90-98%) in the prevention of some HPV-related precancerous diseases has been maintained for up to six years. However, in individuals with impaired reactivity of the immune system, the response of antibodies to active immunization may be reduced [8].

One of the most important factors that determine the body's resistance or sensitivity to HPV is the state of immunological reactivity of the body. As shown by many clinical and immunological studies, persistent chronic infection causes disorders of local and systemic immunity, which significantly affect the clinical course of the disease and the effectiveness of treatment [13]. All this determines the relevance of our study, which aimed to determine the disturbances of immune homeostasis in patients with laryngeal papillomatosis before and at different times after complex treatment with the inclusion in the anti-relapse therapy of quadrivalent antiviral vaccine "Gardasil".

MATERIALS AND METHODS OF RESEARCH

The study of immunological reactivity was performed in 26 patients with recurrent LP before treatment and 21 patients after complex treatment, which included surgery to remove papillomas and a course of antirelapse therapy. The latter consisted of intraoperative administration of aminocaproic acid intravenously, inhalations of laferobion, vitamin-mineral complex (vitamins A, E, C, Se, Zn, Cu) and three injections of quadrivalent Gardasil vaccine into the deltoid muscle of the shoulder, which were administered by the scheme according to which the second and third injections were administered 2 and 6 months after the first.

In the removed pathologically changed tissues of all patients by polymerase chain reaction (PCR) HPV type 11, type 6, their combination or association with high carcinogenic risk of HPV were identified [4]. Patients after treatment were divided into two groups depending on the duration of observation. The first group consisted of patients who were re-examined 2-12 months after treatment, the second group – patients with a re-examination period of more than a year (13-29 months). The results were compared with similar indicators in almost healthy individuals of the control group (n=10).

Determination of systemic immunological reactivity of patients was performed by obtaining serum and isolating blood mononuclear cells in the density gradient of ficoll.-verografin. Indicators of cellular immunity were characterized by quantifying the composition of $CD3^+$-, $CD4^+$- and $CD8^+$- of subpopulations of blood lymphocytes using monoclonal antibodies to lymphocytic CD-markers (manufactured in Gomel, Republic of Belarus) by the method [6], cytotoxic activity of natural killer cells (NKC) – by spectrophotometric method according to O.F. Melnykov, T.A. Zayets [5].

The content of circulating immune complexes (CIC) was evaluated by the method of E.S. Belozerova, T.A. Makarova [2].

The blood interferon system was studied using the method of interferon induction in microquantities of undiluted blood in the modification of I.V. Dzyublyk and co-authors [3]. As an inducer for α-IFN, a vaccine strain of Newcastle disease virus 10 TCID per cell was used, γ-interferon was stimulated with the mitogen phytohemagglutinin (PHA) “Difco” at a concentration of 5-10 μg/ml. The optimal time for stimulation of α-IFN is 24 hours, γ-IFN – 42-72 hours at a temperature of 37°C. Interferon activity was assessed by inhibition of the cytopathic action of the test virus, vesicular stomatitis virus, Indiana strain in Hep-2 cell culture. The optical density was measured at a wavelength of 570 nm [3].

The concentration of immunoglobulins, α-IFN, γ-IFN and TNF-α in blood serum and sIgA in the oropharyngeal secretion was determined by ELISA, according to the manufacturer's instructions (“HEMA Medica”, Russia).

Statistical processing of the research results was performed using the licensed software package STATISTICA 6.1., Using non-parametric U Wilcoxon-Mann-Whitney test, and the method of angular Fisher's transformation. Statistical processing of interferon status data of patients was performed by determining the probability of the mean difference using Student's test [1].

RESULTS AND DISCUSSION

The results of the studies presented in Table 1 indicate that the number of T-lymphocytes in patients with LP is significantly reduced, mainly due to their $CD4^+$ subpopulation. After treatment there was a normalization of the relative number of $CD4^+$ cells in the peripheral blood of patients during the first year after vaccination, which again decreased at

a later date. The relative number of $CD8^+$ lymphocytes in patients with LP probably did not differ from the values of similar parameters in the control group and did not change significantly at different times after treatment.

Indicators of functional activity of natural killer cells play an important role among cellular reactivity reactions in patients with LP both for the course and the prognosis of the disease.

Table 1

Parameters of cellular immunity in patients with LP in different terms of examination

Parameters	Relative number of cells, %			
	before treatment n=13	after treatment		control n=14
		2-12 months n=11	13-29 months n=13	
$CD3^+$ T-lymphcytes, %	45.4* (33.0-60.0)	52.6 (44.0-62.0)	49.2 (36.0-56.0)	54.1 (40.0-65.0)
$CD4^+$ T-lymphocytes, %	28.9** (17.0-46.0)	34.2 (26.0-45.0)	30.8** (21.0-36.0)	35.6 (27.0-41.0)
$CD8^+$ T-lymphocytes, %	19.4 (10.0-31.0)	18.4 (14.0-26.0)	18.6 (13.0-25.0)	21.1 (14.0-28.0)
Activity of NCC, %	22.5 (0.0-63.4)	18.2 (0.0-65.2)	13.4* (0.0-33.9)	23.9 (10.8-32.8)

Notes: in brackets fluctuation limits are presented, * – $p<0.05$; ** – $p<0.01$ as compared with control.

Studies have shown that the average levels of natural cytotoxic activity (NCA) of peripheral blood mononuclear cells in patients with laryngeal papillomatosis did not differ on average from those of almost healthy individuals in the control group (Table 1). However, in patients with LP the ranges of individual fluctuations in the levels of functional activity of NCC are expanded significantly, they ranged from 0 to 63.4%, compared with those in the control group, being 10.8-32.8%, which was the basis for by which we determined the number of patients with normal, reduced or increased level of functional activity of NCC in each group of subjects. After treatment, the average value of NCC activity in the blood of patients in remission during the first year after vaccination tended to decrease. Over a period of observation of more than a year, the average value of NCA levels of blood cells of patients decreased, probably differing from other groups of patients due to the reduction in patients stimulated to treatment of NCA levels to normal values, as indicated in Table 1 limits of individual fluctuations of these indicators. This led to an increase of the relative number of persons with normal values of functional activity of NAC after treatment to 40% during the first year ($p=0.05$) and 38.5% in the subsequent observation as compared with 23.5% of such patients at the initial examination.

The results of the study of interferon status in patients with LP showed significant disorders in the system of interferon-genesis, which were manifested by inhibition of *in vitro* production by blood cells of patients of α- and γ-interferons against the background of stimulation of spontaneous interferon production and increase its serum level (Table 2).

Determination of α- and γ-interferons in the serum of patients in the dynamics of observation showed that the increase in the concentration of serum interferon IFN was accompanied by an increase in γ-IFN at constant levels of α- IFN in the serum of patients with LP, which together with the suppression of blood cells to stimulated production of α-INF indicates a lack of effectiveness of antiviral protection mediated by the interferon system.

After the treatment, it was found that the ability to production of α- and γ-interferons by the stimulated blood cells of patients increased significantly, probably exceeding similar indicators before treatment. In the serum of patients there was an increase in levels of both γ-INF and α-INF (Table 2). Spontaneous production of interferon by blood cells of patients and the level of serum γ-IFN remained increased.

Table 2

Findings of interferon status in patients with LP before and at different terms after treatment (M±m)

Findings	Mean titres of interferon, u/ml			
	control group n=10	patients, before treatment	patients, after treatment	
		n=16	in 2-12 months n=10	in 13-29 months n=13
Spontaneous production of IFN	2.3±2.0	4.0±1.5**	4.0±1.0	4.0±1.5
Production of α- IFN	34.8±2.5	13.4±2.0**	34.5±1.9*	28.7±2.2*
Production of γ- IFN	14.6±1.7	4.3±1.0**	10.8±1.5*	9.4±1.5*
Serum IFN	7.3±1.7	10.4±1.5**	9.3±2.1	9.9±2.0

Notes: * – results statistically different from the ones in patients before treatment, p<0,05; ** – results statistically different from control, p<0.05; n – number of the examined in the group

Indicators of the content of one of the important indicators of activation of the inflammatory process – TNF-α in the serum of patients in the dynamics of treatment indicate a probable increase in its level in patients compared with the control group and a significant decrease after treatment (Table 3).

Table 3

Levels of α-IFN , γ-IFN та TNF- α in blood serum of patients with LP in dynamics of observation

Findings	Groups of study subjects		
	control	Patients with LP	
		before treatment	in 13-29 months after treatment
α- IFN, pg/ml	31.5 (2.0-66.8) n=15	31.1 (0-91.3) n=11	260.0 * (20.7-632.1) n=10
γ –IFN, pg/ml	51.1 (23.5-89.7) n=8	131.6* (14.6-920.7) n=10	219.1* (17.1-958.9) n=9
TNF- α, pg/ml	3.9 (0-11.1) n=8	8.1* (5.6-14.8) n=9	5.7 (4.9-7.4) n=11

Notes: in brackets fluctuation limits are presented; * – p<0.05 as compared with control

The state of humoral defense mechanisms of patients with LP was assessed by determining the content of circulating immune complexes, immunoglobulins of different classes of serum and secretory IgA in the secretion of the oropharynx.

It is shown that the level of circulating immune complexes in patients with LP significantly exceeds the one in the control group (Table 4). After the treatment in 2-12 months the concentration of CIC in the blood of patients with LP decreased and on average did not differ significantly from the indicators in the control group. After the vaccination in 13-29 months CIC values were completely normalized and did not exceed the control values in all examined patients.

Analysis of studies of the content of serum immunoglobulins of different classes shows an increase in the concentration of serum IgM in

patients with LP compared to its content in the control group, and this trend of changes in this indicator remains after treatment. Serum IgG levels in patients before and after treatment did not fluctuate significantly and in most patients did not exceed physiological values. Increased IgA content in patients with LP at the expense of significant increase in the concentration in some patients (6 patients, 28.6%) after treatment decreases almost to the values of the control group.

Table 4

Concentration of immunoglobulins of various classes, CIC in blood serum and secretory immunoglobulin A in oropharynx secretion of patients with LP before and after treatment

Groups of patients	Statistical indicators	IgM, g/l	IgG, g/l	IgA, g/l	CIC, c.u.	sIgA, mg/l
Before treatment	MV	1.5*	11.5	2.6	67.6*	580*
	FL	0.9 – 2.8	7.2 – 15.7	0.7 – 7.6	7.0-155.0	130 – 1275
	n	21	21	21	18	8
2-12 months after treatment	MV	1.4	11.0	1.9	45.7	520
	FL	0.8 – 2.8	9.0 – 15.0	0.5 – 3.2	5.0-95.0	487 – 556
	n	12	12	12	11	3
13-24 months after treatment	MV	1.7	10.9	2.0	37.5	440*
	FL	0.6 – 3.9	7.3 – 13.7	1.2 – 3.4	5.0-61.0	124 – 1104
	n	13	13	13	13	9
Control group	MV	1.1	12.0	1.8	28.0	220
	FL	0.5-1.6	10.5 -5.0	1.0-2.40	0-62.0	58 - 700
	n	10	10	10	15	10

Notes: MV – mean value; FL – fluctuations limits; n – number of study subjects; * – significance of differences relative to control, $p<0.05$.

The amount of secretory IgA in the oropharyngeal secretion of patients with LP probably exceeds the control values in all groups of subjects, changing insignificantly in different periods of observation (Table 5), which indicates a high level of response to viral infection by the main factor of local immunity of mucosal membranes of the oropharynx, playing the role of a compensatory mechanism, especially in conditions of cellular immunity.

Thus, the obtained research results indicate that in patients with LP significant changes in cellular and humoral immunity are detected, which are manifested by a deficiency of peripheral blood T-cells, mainly due to their $CD4^+$-subpopulation, multidirectional changes in the cytotoxic activity of natural killer cells, disorders of interferon status in the form of inhibition of stimulated production of α- and γ-interferons by blood cells and spontaneous production of interferon by blood cells *in vitro*, increased level of serum γ-INF, increased levels of CIC and TNF-α levels. An increase in the concentration of secretory IgA was detected in the oropharyngeal secretion of patients with LP.

The results of repeated studies of immunological reactivity of patients indicate that the treatment, especially during the first year after vaccination, contributed to the normalization of most of the altered factors of the immune system. In particular, there was an increase in the relative number of blood T-cells and their $CD4^+$-subpopulation, normalization of stimulated cytotoxic activity of NCC, the ability to produce *in vitro* of alpha- and gamma interferons by blood cells, the concentration of CIC in blood serum. In both two terms of patients' follow-up after treatment, spontaneous production of interferon by blood cells and serum γ-INF levels remain increased. At the same time, the concentration of antiviral α-INF in the blood serum increases significantly and the content of TNF-α is normalized.

Elevated levels of secretory IgA in the oropharyngeal secretion of patients remained unchanged.

The obtained results testify to the effectiveness of the proposed treatment with using complex of antirelapse therapy vaccine "Gardasil" in the postoperative period, capable of correcting most of the identified disorders of immunological reactivity, against which clinical manifestations of the disease proceed; this is confirmed by positive clinical results – the absence of cases of malignant transformation of the disease during the follow-up period, an increase in the intercurrent period, a probable decrease in their recurrence rate to 4.8% compared with 23.5% of relapses in patients receiving similar treatment except for vaccination ($p\leq0.02$) [7].

As shown by the results of our previous studies conducted by polymerase chain reaction, among 55 patients with LP in removed pathologically altered tissues, HPV type 6 (35%) patients were the most common, in 33% of cases in tissue of removed papillomas, HPV type 11 was detected, in 18% of cases the association of 6 and 11 types of viruses was determined, their combination with human papilloma viruses of high carcinogenic risk – in 14% of patients [3]. Control PCR analysis performed 1 year after vaccination in 10 patients did not reveal the presence of 6, 11 and 16-33 types of HPV in the laryngeal mucosal scraping.

The results of our study correlate with the data of other authors that the vaccines "Gardasil-4" and "Gardasil-9" cause stronger cell-mediated and humoral immune responses to HPV than those that were activated by HPV infection, they are able to stimulate the reactivity of antibodies to associated types of HPV, which is accompanied by a decrease in the frequency of surgical interventions, prolongation of the intercurrent period [9, 15].

The results of clinical studies suggest that modern HPV vaccines can be used in patients with HPV-associated diseases as part of adjuvant therapy, their effectiveness has been confirmed in the treatment of patients with recurrent respiratory papillomatosis [11, 17].

Therewith, changes in a number of indicators of immunological reactivity revealed in the patients after treatment indicate persistent disorders of immune homeostasis which were caused by chronic persistent viral infection and may justify the feasibility of long-term clinical and immunological monitoring of these patients and further search for new effective schemes of adjuvant therapy using modern polyvalent vaccines against HPV in order to increase the effectiveness of their treatment, namely: correction of altered indicators of immune homeostasis, formation of long-term specific protection, prevention of further relapses.

CONCLUSIONS

1. The study of immunological reactivity of patients with LP revealed a disorder of a number of indicators of cellular and humoral immunity which were reflected in multidirectional changes in the cytotoxic activity of natural killer cells, decrease in the relative number of T-lymphocytes mainly due to their CD4+-subpopulation, disorder of interferon status, increased CIC level and the concentration of secretory IgA in the secretion from the oropharynx. The imbalance in the cytokine system of patients was marked by an increase in serum levels of γ-INF and TNF-α, an increase in spontaneous interferon production and suppression of stimulated production of α- and γ-interferons by blood cells in vitro.
2. Comprehensive treatment with quadrivalent vaccine "Gardasil" contributed to the normalization of the most of the altered indicators of immunological reactivity, namely an increase in blood T-lymphocytes and their CD4+-subpopulation, normalization of increased cytotoxic activity of NCA and the ability to stimulate *in vitro* production of α- and γ-interferons by the blood cells, normalization of CIC and TNF-α levels in serum.
3. The results obtained indicate the effectiveness of the Gardasil-4 vaccine in the complex of postoperative therapy of LP patients, as indicated by a significant reduction in the frequency of recurrences, absence of cases of malignant transformation of the disease, normalization of most identified disorders of immunological reactivity and negative results of PCR analysis for HPV determining.

Conflict of interests. The authors declare no conflict of interest.

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